Characterization of the expression and activity of the periplasmic nitrate reductase of Paracoccus pantotrophus in chemostat cultures

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The periplasmic nitrate reductase (Nap) from Paracoccus pantotrophus has a role in cellular redox balancing. Previously, transcription from the nap promoter in P. pantotrophus was shown to be responsive to the oxidation state of the carbon substrate. During batch culture, expression was higher during growth on reduced substrates such as butyrate compared to more oxidized substrates such as succinate. In the present study the effect of growth rate on nap expression in succinate-, acetate- and butyrate-limited chemostat cultures was investigated. In all three cases transcription from the nap promoter and Nap enzyme activity showed a strong correlation. At the fastest growth rates tested for the three substrates nap expression and Nap activity were highest when growth occurred on the most reduced substrate (butyrate > acetate > succinate). However, in all three cases a bell-shaped pattern of expression was observed as a function of growth rate, with the highest levels of nap expression and Nap activity being observed at intermediate growth rates. This effect was most pronounced on succinate, where an approximately fivefold variation was observed, and at intermediate dilution rates nap expression and Nap activity were comparable on all three carbon substrates. Analysis of mRNA prepared from the succinate-grown cultures revealed that different transcription initiation start sites for the nap operon were utilized as the growth rate changed. This study establishes a new regulatory feature of nap expression in P. pantotrophus that occurs at the level of transcription in response to growth rate in carbon-limited cultures.

INTRODUCTION

Paracoccus denitrificans is a nutritionally versatile Gram-negative z-proteobacterium (Berks et al., 1995a; Baker et al., 1998). It can sustain growth under heterotrophic conditions with a range of terminal respiratory electron acceptors including nitrate (NO3−). In this organism the reduction of nitrate to nitrite is the first step of denitrification. In some cases complete denitrification can occur at high dissolved oxygen concentrations, a phenomenon first observed in Paracoccus species (Robertson et al., 1988; Robertson & Kuenen, 1990). Whilst debate exists over which species and strains of Paracoccus denitrify aerobically, the ability to reduce nitrate to nitrite under aerobic conditions is accepted. Aerobic nitrate reduction has been shown to occur in a variety of bacteria (Carter et al., 1995). It is for Paracoccus sp., however, that most is known about aerobic nitrate respiration (Robertson & Kuenen, 1984, 1990; Bell et al., 1990, 1993; Richardson & Ferguson, 1992; Sears et al., 1997). The periplasmic nitrate reductase (Nap) is responsible for the aerobic reduction of nitrate to nitrite. In Paracoccus denitrificans Pd1222 and P. pantotrophus (a close relative of P. denitrificans) the enzyme is considered to provide the cell with an electron-transfer route from NADH which has a submaximal q+/2e− ratio (=4). Compared to the q+/2e− ratio of 10 for the aat3-type oxidase dependent electron transfer pathway, the Nap-dependent pathway allows the cell to dissipate excess reductant under aerobic growth conditions. Such excess reductant could be generated, for example, from the oxidative metabolism of highly reduced carbon substrates such as butyrate (Richardson & Ferguson, 1992; Sears et al., 1993, 1997; Ellington et al., 2002). Examples of other pathways able to re-poise the redox balance of the cell include the reductive assimilatory fixation of CO2, heterotrophic nitrification, the use of poorly coupled oxidases such as the quinol-oxidizing ba3-type oxidase.
oxidase, and polyhydroxyalkanoate biosynthesis (Robertson & Kuenen, 1990; Robertson et al., 1989; Richardson, 2000).

In continuous culture, periplasmic nitrate reductase activity in P. denitrificans Pd1222 was found to be high during carbon-limited growth on butyrate as compared to the more oxidized substrate malate. Expression was observed at all oxygen concentrations tested between 0 and 100% air saturation (Sears et al., 1997). Nap activity in batch cultures of P. pantotrophus and P. denitrificans Pd1222 has been found to be comparable. In response to aerobic growth on the reduced carbon substrate butyrate, Nap activities increase by at least an order of magnitude, compared to levels seen with relatively oxidized substrates such as malate or succinate (Richardson & Ferguson, 1992; Sears et al., 1993, 1995). Further studies in P. pantotrophus have shown transcription to occur differentially from two transcriptional initiation start sites upstream of napE in response to carbon substrate and oxygen (Sears et al., 2000). The upstream site, P1, is utilized at a low level during aerobic growth on succinate, whilst the downstream site, P2, is utilized more during aerobic growth on butyrate. During anaerobic growth both P1 and P2 are inactive. Quantification of nap expression in batch cultures of P. pantotrophus (nap–lacZ) has demonstrated expression to be positively responsive to oxygen and to be tightly regulated in response to the carbon substrate. Correlation between Nap activity and nap expression has demonstrated transcription from the nap promoter to be the major point of regulation for cellular Nap activity (Richardson & Ferguson, 1992; Ellington et al., 2002). In these batch culture experiments transcription appeared to be independent of growth phase, since activity reached a maximum during the exponential phase of growth and did not increase during the deceleration or stationary phases of growth (Ellington et al., 2002). However, there was an inverse relationship between the maximum specific growth rate ($\mu_{max}$) during exponential phase and nap expression. Thus $\mu_{max}$ decreased in the order succinate > acetate > butyrate, whilst levels of nap expression increased in the same order, succinate < acetate < butyrate. In light of this, the present study sought to assess the effect of growth rate ($\mu$) on nap expression independently of batch culture growth phase. The effect of the dilution rate ($D$; which is equivalent to $\mu$ under steady-state conditions) on nap expression and Nap activity in carbon-limited chemostat cultures is reported.

**METHODS**

**Bacterial culture conditions.** Paracoccus pantotrophus (nap–lacZ) (Ellington et al., 2002), containing a plasmid-borne transcriptional fusion of the napE promoter region to the lacZ gene, was used for study of transcriptional regulation. The nap–lacZ fusion includes nucleotides 826 to 1035 of the published nap sequence (Berks et al., 1995b). The isolate was grown at 30°C in the minimal medium described by Robertson & Kuenen (1983), with succinate or butyrate added to 5 mM, or acetate added to 7.5 mM in continuous culture with 25 mM KNO$_3$ added. Batch cultures contained 30 mM succinate, 50 mM acetate or 10 mM butyrate. Antibiotics were added at the following concentrations: streptomycin, 50 µg ml$^{-1}$; spectinomycin, 50 µg ml$^{-1}$; gentamicin, 10 µg ml$^{-1}$. For batch cultures 250 ml shake flasks containing 50 ml medium were used. Periodically, 1 ml samples were removed and culture turbidity at 600 nm was measured. Plots of log OD$_{600}$ versus time were used to determine $\mu_{max}$ from the gradient of the exponential growth phase. The continuous culture vessel contained a working volume of 1-5 l. Typically, a 1% (v/v) inoculum from overnight cultures was used, and the vessel was operated in batch mode until the culture reached OD$_{600}$ 0-8-1-0. Once the culture was in the late exponential phase of growth the vessel was switched to continuous mode at the desired $D$ (h$^{-1}$). pH and dissolved oxygen concentrations were monitored with a combined glass electrode (Ingold) and a polarographic PO$_2$ electrode (Ingold), respectively. pH was observed to be between 6-6 and 8-2 during operation. Dissolved oxygen concentration was maintained by sparging air into the culture vessel; concentrations were observed to be between 85 and 100% air saturation. In order to ensure no contamination had occurred, cultures were checked by microscopy and streaking on to agar plates.

The culture was allowed at least 4 vessel-volume changes before being judged to be at steady state. To check for carbon limitation, culture supernatants were analysed using a Dionex anion-exchange HPLC chromatography system, whereby known concentration standards were used to quantitate substrate concentration. Elution profiles revealed each carbon substrate to be undetectable. Moreover, at the end of operation of each chemostat an addition of 10 mM carbon substrate was made, and cultures were seen rapidly to increase their biomass to double or more of that seen previously under carbon-limited conditions.

**Analytical procedures.** Culture samples of 5, 10, 20 or 50 ml were withdrawn through a small-bore tube attached to a sample bottle. Culture samples of 5 ml were placed on ice for immediate determination of nitrite concentration and β-galactosidase activity. Cells from 2 × 10 ml, 2 × 20 ml and 50 ml samples were harvested by centrifugation at 10000 g. The 50 ml sample was resuspended in 500 µl 50 mM phosphate buffer pH 7.0, dried overnight at 80°C and weighed for biomass determination. Cell pellets and supernatants from the two 20 ml samples were frozen separately at −80°C. Cell pellets were used to prepare DNA and RNA for relative plasmid copy number and primer extension analysis, respectively. Supernatants from these samples were used for HPLC determination of the residual carbon source and nitrate concentrations in the reservoir medium. The 10 ml samples were utilized for protein determination and methylviologen assays.

**NO$_3^-$ assays.** Residual nitrate concentration was measured using a Dionex anion-exchange HPLC system with a UV detector, whereby a standard of known concentration was used to facilitate the quantification of nitrate concentration. Nitrite concentrations were determined spectrophotometrically by a colorimetric change assay (Coleman et al., 1978). From assays of nitrate concentration in the culture vessel at steady state (above) nitrite accumulation rates were calculated and determined as a function of biomass at steady state.

**Enzyme activity.** Periplasmic nitrate reductase activity was determined spectrophotometrically using reduced methylviologen (MV$^+$) as the electron donor (Sears et al., 1993). Absorption spectra were collected using a Varian spectrophotometer. The difference between starting and steady-state concentrations of nitrate was used to calculate the steady-state rates for nitrate reduction, which were given as a function of the steady-state biomass of the culture.

**Transcription analysis.** β-Galactosidase activity from nap–lacZ (Ellington et al., 2002) was determined spectrophotometrically according to Miller (1972) using 0-1 ml of culture sample. RNA
samples isolated from 20 ml cell culture using an RNeasy RNA isolation kit (Qiagen) were used for primer extension analysis. This was carried out exactly as described previously (Sears et al., 2000).

**Protein determination.** A cell pellet from 10 ml of cell culture was used for total cell protein determination (see above). The cell pellet was resuspended in 0.5 ml 0.7 M NaOH and heated in a boiling water bath for 10 min. After cooling for 5 min, 50 μl aliquots were added to 1.95 ml 0.1 M phosphate buffer pH 7.0, and mixed with 1 ml 0.25% CuSO₄ in 10 M NaOH. A Varian spectrophotometer was used to determine the ΔA₉₀₀ after a 10 min incubation at 25°C and protein determination was made against a BSA standard curve.

**RESULTS**

**Effect of carbon substrate and dilution rate on the growth yield of *P. pantotrophus* (nap–lacZ) during aerobic carbon-limited growth**

Growth characteristics of *P. pantotrophus* (nap–lacZ) were analysed in order to determine the effect of dilution rate and carbon substrate during carbon-limited continuous culture. Maximum specific growth rates (μ̂ₘₐₓ) for batch cultures of *P. pantotrophus* (nap–lacZ) were determined during exponential phase and were higher for cultures growing on succinate (0.7 h⁻¹) as opposed to acetate (0.55 h⁻¹) and butyrate (0.15 h⁻¹). Under steady-state conditions in a chemostat, D is considered to be equivalent to μ. However, because a substrate is growth-limiting μₘₐₓ cannot be achieved and washout occurs as D approaches this value. Consequently, for the purpose of this study, cultures limited by succinate and acetate were maintained over a wide range of comparable dilution rates (D=0.036–0.500 h⁻¹), but the butyrate-limited cultures had to be maintained over a distinct and narrower range (D=0.036–0.100 h⁻¹). This makes direct comparisons of the physiology at equivalent D difficult for butyrate-limited compared to succinate-limited and acetate-limited cultures, and the terms high, low and intermediate D will be used to reflect the D relative to μₘₐₓ for each carbon substrate. Thus, with succinate and acetate as the carbon substrate D=0.036 to 0.133 h⁻¹ and 0.500 h⁻¹ are termed low, intermediate and high D, respectively; with butyrate as the carbon substrate D=0.036 h⁻¹, 0.056 h⁻¹ and 0.100 h⁻¹ are termed low, intermediate and high D, respectively.

Biomass increased proportionally to D, regardless of the carbon substrate. In succinate-limited and acetate-limited cultures dry weight increased by approximately twofold over the D range 0.036–0.500 h⁻¹ (Fig. 1a, b). In butyrate-limited cultures the same trend was observed, though with higher absolute values, over the D range 0.036–0.100 h⁻¹ (Fig. 1c). Protein concentrations also increased with the dilution rate, regardless of the growth-limiting carbon substrate (Fig. 1a–c). Conversely, relating the total protein content to the biomass of the culture (percentage of biomass as protein) reveals an inverse relationship to the dilution rate (Fig. 1a–c). In succinate-limited and acetate-limited cultures of *P. pantotrophus* (nap–lacZ) the protein as a percentage of dry weight decreased by 10% between D=0.036 h⁻¹ and 0.500 h⁻¹ (Fig. 1a, b). In butyrate-limited cultures a decrease of 25% was observed between D=0.036 h⁻¹ and 0.100 h⁻¹ (Fig. 1c). An inverse relationship between the percentage of biomass as protein and D is a commonly observed feature in carbon-limited bacterial chemostats (Harder & Dijkhuizen, 1982).

During carbon-limited growth on all three substrates increase in D resulted in increased growth yields (Fig. 1a–c). To investigate the effect of D on the cellular fate of available carbon, maintenance carbon consumption rates (qₘₐₓ) and ‘true’ growth yields (Yₛ) observed growth yield corrected for maintenance energy losses) were calculated (Table 1). During limitation by all three carbon substrates growth yield losses due to maintenance requirements were inversely proportional to D (Table 1). For succinate- and acetate-limited cultures at the highest D, approaching μₘₐₓ the observed growth yield (Yₒᵇₛ) approached Yₛ. Conversely, Yₒᵇₛ was twofold lower than Yₛ at the lowest D studied for both substrates. This suggests that at low D a large proportion of the available carbon was utilized for maintenance functions. The analysis of Yₒᵇₛ showed that the...
Table 1. Growth yield analysis of *P. pantotrophus* (nap–lacZ) cultures limited by succinate, acetate and butyrate

The maintenance rate (*q_m*) and true growth yield (*Y_g*) were determined from a double reciprocal plot according to the equation 1/Y_red = 1/Y_g + q_m/μ. Values for low, intermediate and high dilution rates were taken from *D* = 0·036 h⁻¹, 0·133 h⁻¹ and 0·500 h⁻¹ respectively for succinate- and acetate-limited cultures, and *D* = 0·036 h⁻¹, 0·056 h⁻¹ and 0·100 h⁻¹ respectively for butyrate-limited cultures.

<table>
<thead>
<tr>
<th>C. source</th>
<th><em>q_m</em> (μmol mg⁻¹ h⁻¹)</th>
<th><em>Y_g</em> (g mol⁻¹)</th>
<th><em>Y_red</em> (g mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>0·328</td>
<td>87</td>
<td>49</td>
</tr>
<tr>
<td>Acetate</td>
<td>0·506</td>
<td>65</td>
<td>34</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0·420</td>
<td>247</td>
<td>68</td>
</tr>
</tbody>
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Nitrate consumption and steady-state nitrite concentration at various dilution rates in aerobic cultures of *P. pantotrophus* (nap–lacZ) limited by succinate, acetate or butyrate

The rate of nitrate consumption was dependent on the carbon substrate and the dilution rate. At the highest *D* monitored for each carbon substrate, the rates of nitrate consumption increased in the order succinate (30)< acetate (70)< butyrate (200) [numbers in parentheses represent rates in units of nmol nitrate (mg dry weight)⁻¹ h⁻¹]. In all cases the rates of nitrate consumption increased at intermediate *D* and decreased again at low *D* (Fig. 2a–c). Since ammonium was present in excess under all growth conditions it is likely that this nitrate consumption reflects aerobic nitrate respiration in which nitrate is reduced to nitrite by Nap, rather than being consumed by nitrate assimilation. This view was supported by the observed steady-state concentration of extracellular nitrite (Fig. 2a–c). The steady-state rate of nitrite accumulation was always lower than the steady-state rate of nitrate consumption. This suggested that aerobic nitrite reduction was also occurring in these cultures.

Effect of dilution rate on Nap activity in *P. pantotrophus* (nap–lacZ), during aerobic carbon-limited growth

To assess if a Nap enzyme was present in the aerobic culture which could account for nitrate consumption, methylviologen assays specific for periplasmic nitrate reductase activity were used to determine cellular Nap activity in *P. pantotrophus* (nap–lacZ). During growth on all carbon substrates Nap activity was responsive to the dilution rate. At the highest *D* monitored for each substrate Nap activity increased in the order succinate (7·5)< acetate (20)< butyrate (40) [numbers in parentheses represent Nap activity in units of nmol methylviologen oxidized (mg dry weight)⁻¹ mg⁻¹]. This pattern has previously been observed in batch cultures (Richardson & Ferguson, 1992). Regardless of carbon substrate, Nap activity maxima were observed at intermediate *D*; nevertheless, the specific activity of butyrate cultures was still twofold higher than that of succinate cultures (Fig. 3a–c). The effect of *D* was most pronounced in the succinate-limited culture, where a threefold variation in Nap specific activity as a function of *D* was apparent (Fig. 3a).
Nap expression in *P. pantotrophus* chemostats

![Fig. 3](image)

**Fig. 3.** Effect of dilution rate on Nap activity and β-galactosidase activity in (a) succinate-, (b) acetate- and (c) butyrate-limited aerobic chemostat cultures of *P. pantotrophus* (nap–lacZ).

■ Specific nitrate reductase activity; △ β-galactosidase activity, measuring transcription from the nap promoter. Dilution rates were 0·036 h⁻¹, 0·076 h⁻¹, 0·133 h⁻¹, 0·250 h⁻¹ and 0·500 h⁻¹ for succinate- and acetate-limited cultures, and 0·036 h⁻¹, 0·056 h⁻¹, 0·076 h⁻¹ and 0·100 h⁻¹ for butyrate-limited cultures.

**Effect of carbon substrate on transcription from the nap promoter during aerobic carbon-limited growth in *P. pantotrophus* (nap–lacZ)**

It should be noted that the Nap activity measured in the previous section is a consequence of transcription and translation of the chromosomal nap genes followed by Nap cofactor assembly and export of the holoenzyme. To study the first of these processes, transcription, in more detail β-galactosidase activity of *P. pantotrophus* (nap–lacZ) was measured. At the highest D monitored for each substrate nap expression increased in the order succinate (2500) < acetate (4000) < butyrate (8500) (numbers in parentheses represent β-galactosidase activity in Miller units). This pattern has previously been observed in batch cultures, where expression of the nap promoter has been found to be low, at 500 units of β-galactosidase activity, during growth on succinate, and higher, at 4500 units of β-galactosidase activity, during growth on butyrate (Ellington et al., 2002). To establish if any variations in nap expression were the result of a plasmid copy number effect, plasmid mini-preparations from each sample were made, digested with SalI and electrophoresed on a 0·8 % TAE agarose gel. Loadings were compensated for mg dry wt⁻¹ to ensure a direct comparison of relative plasmid copy number per cell between samples. Densitometric analysis of the plasmid DNA bands revealed no significant differences, indicating that the plasmid copy number per cell did not change significantly in response to the carbon substrate or D (data not shown).

Expression from the nap promoter responded to the D and the carbon substrate in a similar pattern to that observed for Nap activity in *P. pantotrophus* (nap–lacZ) (Fig. 3a–c). Expression maxima occurred at intermediate D on each carbon substrate such that the maximal β-galactosidase activities for each carbon-limited culture were comparable. The largest D-dependent variation in β-galactosidase activity was a fivefold difference, which occurred in response to D during succinate-limited growth.

Whilst the above data indicate that D and carbon substrate can affect Nap regulation independently, the largest variation in nap expression resulted from the combined effects of D and carbon substrate. For example, a sixfold difference is apparent between succinate-limited cultures at high D and butyrate-limited cultures at intermediate D (Fig. 3). This suggests that nap regulation is affected by a physiological signal which integrates aspects of growth rate and carbon substrate utilization.

The β-galactosidase activity data suggested that D affected transcription from the nap promoter independently of the carbon substrate. This effect was very marked in succinate-limited cultures and is in clear contrast to the situation in batch cultures utilizing succinate, where transcription levels are comparatively low throughout all the growth phases (Ellington et al., 2002). To study this phenomenon in more detail in the succinate-limited chemostat cultures, primer extension analysis was undertaken. Previous work has established the differential use of two transcription initiation sites in batch culture in response to the carbon substrate. The upstream site P1 is used weakly during growth on succinate, when low enzyme and promoter activity occur, whilst the downstream P2 site is used more during growth conditions where high levels of enzyme and promoter activity occur (Sears et al., 2000; Ellington et al., 2002). Primer extension analysis of total RNA was used to investigate if transcription start site utilization altered in response to the dilution rate in aerobic succinate-limited continuous cultures. The technique was also used to compare the pattern of transcription initiation in continuous culture with that seen in aerobic batch cultures with succinate or butyrate as the carbon substrate. This comparison indicates that the two transcriptional initiation start sites, P1 and P2, previously identified under batch culture conditions, are also utilized under the continuous culture conditions used in this study. During aerobic succinate-limited continuous culture, transcription initiated relatively weakly from only the P1 site at the lowest and
highest dilution rates, 0·036 h⁻¹ and 0·500 h⁻¹, respectively. At the intermediate dilution rate 0·133 h⁻¹, where Nap expression was highest, transcription was also initiated from the P2 site (Fig. 4). This indicates that the differential utilization of the transcription start sites, described previously in response to carbon substrate (Sears et al., 2000), can also occur in response to the dilution rate and independently of carbon substrate.

**DISCUSSION**

This study has examined the activity and expression of the periplasmic nitrate reductase of *P. pantotrophus* during carbon-limited growth with succinate, acetate or butyrate as sole carbon source. The influence of *D* was also examined. At each *D*, the nap system was studied at the level of gene expression, levels of active enzyme and electron flux through Nap. The results reveal a new aspect of the regulation of the periplasmic nitrate reductase in *P. pantotrophus* in which expression and *in vivo* activity of the enzyme are dependent on *D*, in addition to the carbon source being utilized.

The analysis of cellular carbon partitioning in *P. pantotrophus* revealed that a greater rate of carbon consumption for cellular maintenance occurred during growth on butyrate than during growth on succinate. The highest maintenance rate was observed during growth on acetate. This is unsurprising, as acetate is a two-carbon substrate which must be metabolized via the glyoxylate cycle or bypass, requiring an input of four carbons, or two molecules, to complete a cycle. In contrast to this, butyrate is a four-carbon substrate. However, whilst it must also be metabolized via the glyoxylate cycle, a cycle can be completed with one molecule. If the two-carbon nature of acetate is accounted for and the maintenance requirement halved from 506 μmol mg⁻¹ h⁻¹ to 253 μmol mg⁻¹ h⁻¹ then butyrate has the highest maintenance requirement. The observed high culture maintenance rate and slow growth rate observed in batch (Ellington et al., 2002) and continuous culture indicates the challenge that the metabolism of butyrate for growth poses to the cell.

The results show that aerobic nitrate reduction occurs under all the carbon-limited continuous culture conditions tested. Furthermore, nitrite accumulation did not match nitrate consumption, indicating that, as observed previously (Sears et al., 1997), nitrite reduction had also occurred. The proportion of succinate oxidation linked to the reduction of nitrate under aerobic conditions at the intermediate dilution rate *D*=0·133 h⁻¹ is small. The steady-state reduction rate of nitrate is 21 μmol l⁻¹ h⁻¹ (calculated from Figs 1 and 2). From equation 1 it is apparent that complete oxidation of 1 mol succinate could provide reduction for 7 mol nitrate:

\[
\text{C}_4\text{H}_8\text{O}_4 + 7\text{NO}_3^- \rightarrow 4\text{CO}_2 + 3\text{H}_2\text{O} + 7\text{NO}_2^- \quad (1)
\]

Therefore, a nitrate reduction rate of 21 μmol l⁻¹ h⁻¹ accounts for only 3 μmol succinate l⁻¹ h⁻¹. This is a small fraction of the carbon substrate influx given that the rate of succinate flux into the culture vessel is 665 μmol l⁻¹ h⁻¹. At the intermediate *D*=0·056 h⁻¹ during butyrate-limited growth, the proportion of butyrate oxidation linked to nitrate reduction can reach higher levels. Equation 2 shows that complete oxidation of 1 mol of butyrate can provide reduction for 10 mol nitrate:

\[
\text{C}_4\text{H}_8\text{O}_2 + 10\text{NO}_3^- \rightarrow 4\text{CO}_2 + 4\text{H}_2\text{O} + 10\text{NO}_2^- \quad (2)
\]

During butyrate-limited growth the highest rate of nitrate reduction was 204 μmol butyrate l⁻¹ h⁻¹ (calculated from Figs 1 and 2). From equation 2 this can account for the oxidation of 20·4 μmol l⁻¹ h⁻¹. This figure is around 7% of the 280 μmol l⁻¹ h⁻¹ influx of butyrate at *D*=0·056 h⁻¹. The large difference in the proportions of carbon substrate being oxidized by nitrate reduction indicates the importance of nitrate as an auxiliary oxidant during growth on butyrate.

Detailed molecular studies of the regulation of Nap systems

![Fig. 4. Primer extension analysis of pnap–lacZ expression during aerobic continuous succinate-limited growth with variation in D and aerobic succinate and butyrate batch culture growth of P. pantotrophus (pnap–lacZ). Equivalent aliquots of primer extension reactions were applied to a 6% denaturing polyacrylamide gel. RNA samples were isolated from succinate-limited chemostat samples at D=0·036 h⁻¹, 0·133 h⁻¹ and 0·500 h⁻¹, and from aerobic butyrate (But + O₂) and succinate (Succ + O₂) batch cultures during mid-exponential-phase growth, respectively. P1 and P2 initiated transcripts are annotated.](image-url)
have been undertaken in *Rhodobacter* sp. and *Escherichia coli* (Darwin *et al.*, 1998; Dobao *et al.*, 1994; Gavira *et al.*, 2002; Liu *et al.*, 1999; Reyes *et al.*, 1998; Stewart *et al.*, 2002; Wang *et al.*, 1999). A notable feature of Nap systems is the variation, both within and between organisms, in the physiological functions, as well as the regulation and expression, of *nap*. For example, Nap plays a role in anaerobic denitification in *Pseudomonas* G-179 and *Rhodobacter sphaeroides* f. sp. *denitrificans*, in anaerobic nitrate respiration under nitrate-limited conditions in *E. coli*, and redox-poising of the photosynthetic electron-transport chain or survival during light-dark transitions in *Rhodobacter capsulatus* (Jones *et al.*, 1990; Siddiqui *et al.*, 1993; Castillo *et al.*, 1996; Zumft, 1997; Liu *et al.*, 1999; Moreno-Vivian *et al.*, 1999; Philippot & Hojberg, 1999; Potter *et al.*, 1999, 2001; Richardson *et al.*, 2001; Brondijk *et al.*, 2002; Ellington *et al.*, 2003). Previously, the importance of transcriptional regulation for the regulation of Nap activity in *P. pantotrophus* has been established in batch culture (Ellington *et al.*, 2002). The evidence presented in this study supports the assertion that transcriptional regulation is the major point of differential regulation for the *P. pantotrophus* Nap system. Previously, growth on succinate has shown low levels of transcription from the *nap* promoter under all conditions tested. Furthermore, differential transcriptional start site usage and *nap* promoter activity has been found only in response to differing carbon substrates (Sears *et al.*, 2000; Ellington *et al.*, 2002). A new feature of *nap* transcriptional regulation highlighted in this study is differential transcriptional start site usage and *nap* promoter activity in response to the growth rate.

Under the carbon-limited conditions in this study, levels of expression did not fall to those seen in batch culture on any carbon substrate. This suggests that during carbon-limited continuous culture the signal affecting *nap* transcription is more abundant than during batch culture with the same substrates. Together with the effect of growth rate on Nap activity and transcription in carbon-limited cultures this observation reinforces the suggestion that *nap* regulation is affected by a signal originating from the metabolism of a substrate. It also suggests that the abundance of this signal can be altered by the growth rate of *P. pantotrophus* in carbon-limited, continuously growing cultures.

The increased level of transcription and enzyme activity at intermediate growth rates described in this study is a common response of protein synthesis and enzyme activity to growth rate (Harder & Dijkhuizen, 1982). In the case of Nap this phenomenon may be a result of differential reductant accumulation in response to the growth rate of the culture. During carbon-limited growth at low growth rates only a relatively small amount of carbon is available for growth. This could translate into relatively small amounts of reductant accumulating. Likewise, at high dilution rates reductant may not accumulate as readily due to a shift in the equilibrium between oxidized and reduced reducing equivalents as the biosynthesis of new cellular material acts as a sink for excess reductant. However, at intermediate dilution rates sufficient carbon may be available to generate a quantity of excess reducing equivalents. At intermediate growth rates this pool of excess reducing equivalents would not be utilized in biosynthetic reactions.

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**REFERENCES**


