Fine-tuned regulation by oxygen and nitric oxide of the activity of a semi-synthetic FNR-dependent promoter and expression of denitrification enzymes in *Paracoccus denitrificans*

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In *Paracoccus denitrificans* at least three fumarate and nitrate reductase regulator (FNR)-like proteins [FnrP, nitrate and nitric oxide reductases regulator (NNR) and NarR] control the expression of several genes necessary for denitrifying growth. To gain more insight into this regulation, β-galactosidase activity from a plasmid carrying the lacZ gene fused to the *Escherichia coli* melR promoter with the consensus FNR-binding (FF) site was examined. Strains defective in the fnrP gene produced only very low levels of β-galactosidase, indicating that FnrP is the principal activator of the FF promoter. Anoxic β-galactosidase levels were much higher relative to those under oxic growth and were strongly dependent on the nitrogen electron acceptor used, maximal activity being promoted by N2O. Additions of nitrate or nitroprusside lowered β-galactosidase expression resulting from an oxic to micro-oxic switch. These results suggest that the activity of FnrP is influenced not only by oxygen, but also by other factors, most notably by NO concentration.

Observations of nitric oxide reductase (NOR) activity in a nitrite-reductase-deficient strain and in cells treated with haemoglobin provided evidence for dual regulation of the synthesis of this enzyme, partly independent of NO. Both regulatory modes were operative in the FnrP-deficient strain, but not in the NNR-deficient strain, suggesting involvement of the NNR protein. This conclusion was further substantiated by comparing the respective NOR promoter activities.

**INTRODUCTION**

*Paracoccus denitrificans* is a facultative anaerobe capable of reducing nitrate to dinitrogen gas via nitrite, NO and N2O in order to generate metabolic energy in the absence of molecular oxygen. The synthesis of four enzymes involved in denitrification is controlled at the transcriptional level and depends upon the availability of oxygen and nitrogenous electron acceptors (Baker *et al.*, 1998). At least three proteins belonging to the superfamily of fumarate and nitrate reductase (NAR) regulator (FNR)-type transcription factors seem to play an especially important role in this environmental adaptation process. The FnrP protein activates anaerobic expression of the membrane-bound NAR (van Spanning *et al.*, 1997). As is the case for the *Escherichia coli* FNR protein, the N-terminal domain of FnrP harbours three cysteine residues which, together with a cysteine residue located in the central domain, can carry a labile [4Fe–4S] cluster (Hutchings *et al.*, 2002). The remaining proteins, nitrite and nitric oxide reductases regulator (NNR) and NarR, differ markedly from FnrP in that they lack the N-terminal cysteine ligands. NNR is involved in induction of genes in the nir and nor operons encoding nitrite reductase (NIR) and nitric oxide reductase (NOR), respectively (van Spanning *et al.*, 1995), while NarR elevates the expression of NAR in response to nitrate and/or nitrite (Wood *et al.*, 2001).

As yet we do not know exactly how the biological activity of the FNR-like proteins of *P. denitrificans* is modulated. The [4Fe–4S] cluster of the *E. coli* FNR protein exhibits great sensitivity to molecular oxygen, which rapidly converts it to the [2Fe–2S] form in a direct reaction; this is accompanied by dissociation of the FNR dimer into monomers with a loss of the ability to bind DNA (Khoroshilova *et al.*, 1995, 1997; Green *et al.*, 1996; Jordan *et al.*, 1997). An analogous Fe-S cluster could be reconstituted into the overexpressed FnrP in vitro, confirming that this protein...
is a true orthologue of FNR from *E. coli* with a similar mechanism of oxygen sensing (Hutchings et al., 2002). By studying the β-galactosidase activity in *P. denitrificans* strains with the *nitS* and *norC* promoters fused to the *lacZ* reporter gene, NNR-dependent transcription was shown to be controlled by NO or a related species (van Spanning et al., 1999), analogous to earlier findings in *Rhodobacter sphaeroides* (Tosques et al., 1996; Kwiatkowski et al., 1997). Primer extension analysis of the *nor* promoter region revealed the presence of two transcription start sites. A transcript that initiated downstream of the putative NNR-binding site was identified as the major contributor to NNR-dependent anaerobic *nor* expression. Besides this, a weak transcription from an upstream start site still occurred both aerobically and anaerobically (Hutchings & Spiro, 2000).

An understanding of the regulatory network in *P. denitrificans* was initially developed, based upon the use of an artificial FF-*melR* class II promoter, in which the consensus FNR-binding (FF) site is centred at −41·5 with respect to the transcription start site (Lodge et al., 1990). This promoter could be activated in *P. denitrificans* during the switch from oxic to anoxic growth conditions (Spiro, 1992), provided that the culture contained a sufficient amount of iron (Kučera & Mat’chová, 1997). An alternative mode of FF-*melR* activation upon the addition of respiratory inhibitors could be activated in *P. denitrificans* that modulate the extent of this activation?

The second objective of the present study concerns the true role of nitrogenous electron acceptors in the expression of denitrification enzymes. The newly proposed essential activation of NNR by NO has to be reconciled with previous studies showing that oxygen limitation alone was the dominant regulatory factor (Kučera et al., 1984; Boubliková et al., 1985). A possible trivial explanation for the previous results could be the presence of traces of nitrate in the culture media used. To check this possibility, we followed the formation of NOR in cells under carefully controlled growth conditions, minimizing the formation of endogenous NO.

### METHODS

**Strains and growth conditions.** The pertinent bacterial stains used in this study are listed in Table 1. *P. denitrificans* strains were grown at 30°C on a basal salts medium with 50 mM succinate as carbon source (Kučera et al., 1990). When required, rifampicin, tetracycline and kanamycin were added to concentrations of 20, 1 and 30 µg ml⁻¹, respectively. Growth under oxic conditions was carried out with shaking at 200 r.p.m. in 250 ml or 1 litre open conical flasks filled to one-tenth of their volume. Anoxic growth proceeded statically in sealed flasks previously sparged with oxygen-free argon for 5 min; the terminal electron acceptors used were nitrate (10 mM), nitrite (5 mM) or N₂O (sparging with N₂O gas for 5 min). The growth of cells was followed by measuring OD₆₀₀ (1 cm light path). An OD₆₀₀ of 1·0 corresponds to 0·31 mg dry wt (1 culture)⁻¹. Micro-oxic incubation was performed by suspending the oxically grown cells in 50 ml growth medium supplemented with 0·6 mM sodium molybdate at 1·15–1·25 mg dry wt ml⁻¹ and agitating the suspension in 250 ml open conical flasks at 120 r.p.m.

**Table 1. Bacterial strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. denitrificans</em></td>
<td>Pd1222</td>
<td>DSM413 derivative; enhanced conjugation frequencies; RifR</td>
</tr>
<tr>
<td></td>
<td>Pd29.21</td>
<td>Pd1222 derivative; <em>fnrP</em>::KanR</td>
</tr>
<tr>
<td></td>
<td>Pd29.21::Pr811</td>
<td>Pd29.21 derivative; P<em>onorC-lacZ</em> integrated</td>
</tr>
<tr>
<td></td>
<td>Pd71.21</td>
<td>Pd1222 derivative; <em>nitS</em>::KanR</td>
</tr>
<tr>
<td></td>
<td>Pd77.21</td>
<td>Pd1222 derivative; <em>nnr</em>::KanR</td>
</tr>
<tr>
<td></td>
<td>Pd92.30</td>
<td>Pd29.21 derivative; <em>fnrP</em>::KanR; <em>nnr</em> truncated</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>SM10</td>
<td>thr leu hsd recA KanR RP4-2-Tet::Mu integrated</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pPr811</td>
<td>pBK11 derivative; P<em>onorC-lacZ</em></td>
</tr>
<tr>
<td></td>
<td>pRW2FF</td>
<td>TetR IncP (16-9 kb); lacZYA under FNR-dependent promoter</td>
</tr>
</tbody>
</table>

*KanR*, kanamycin resistance; RifR, rifampicin resistance; TetR, tetracycline resistance.
Plasmid isolation and transfer. The bacterial plasmid used was pRW2A/FF, a derivative of the broad-host-range plasmid pRK250 carrying a fusion of the medR promoter to lacZ downstream of an FNR-binding sequence (Table 1). It was purified on a silica column (Qiagen) and then transformed into E. coli SM10 by electroporation as described by Ausubel et al. (1995) by using a Gene Pulser II (Bio-Rad) at a field strength of 2.5 kV cm⁻¹. The plasmid-containing strain E. coli SM10 served as a donor in agar-plate matings with P. denitrificans strains. The donor culture was grown in Luria–Bertani (LB) medium with tetracycline (15 μg ml⁻¹) to stationary phase; the recipient culture was grown in LB with rifampicin (20 μg ml⁻¹) to late exponential phase. Cells were harvested by centrifugation, resuspended in LB, mixed on LB plates at a donor/recipient ratio of 1:10 and incubated at 30 °C for 16 h. After mating, the cells were washed from the agar surface in sterile 0.9% NaCl, diluted appropriately and plated on LB agar supplemented with rifampicin (40 μg ml⁻¹), neomycin (30 μg ml⁻¹; not added to the wild-type culture) and tetracycline (1 μg ml⁻¹) to obtain the exconjugants.

Consideration of the amount of plasmid DNA in cells. Because a plasmid system was used to study FnRP-dependent transcription, we considered the possibility that the various levels of β-galactosidase would emerge as a function of the varying plasmid copy number. Therefore, the amount of plasmid DNA per unit of total cellular protein was estimated for several culture samples and correlated with the corresponding specific activity of β-galactosidase. The bacterial plasmid used was Pd92.30 (Miller units) and then centrifuged.

Enzyme assays. NAR activity was measured spectrophotometrically by following the oxidation of reduced methyl viologen coupled to the reduction of 10 mM nitrate to nitrite in cells made permeable by including 0.1% Triton X-100 in the reaction mixture (Kucéra & Kaplan, 1996). NIR activity was measured by determining the amount of nitrite consumed by a suspension of intact cells in the presence of 20 mM succinate as electron donor (Kucéra et al., 1990). NOR activity was measured amperometrically using a Clark-type electrode sensitive to NO. The reaction mixture contained 5 mM ascorbate in conjunction with 0.2 mM N,N,N′,N′-tetramethylphenylene-1,4-diamine as electron donor and redox mediator, respectively; the initial concentration of NO being 50 μM (Kucéra, 1992). When the sensitivity to antimycin was examined, the artificial electron donor was replaced by 7 mM succinate.

RESULTS

FF-lacZ expression in the fnrP and nrr mutant strains

The primary aim of this work was to determine which of the FNR-type transcription factors mediates the anoxic activation of the FF site in P. denitrificans. For this analysis, plasmid pRW2A/FF was conjugated into fnrP, nrr and fnrP/nrr mutant strains and the cells were harvested from oxically grown cultures shifted to incubation under microoxic conditions in the presence of nitrate. As shown in Table 2, an increase in β-galactosidase reporter activity was apparent in the nrr mutant strain, which contains an intact fnrP gene. On the other hand, much lower transcriptional activation of the FF site took place in the fnrP mutant, in spite of the fact that NNR was probably present and active as shown by the concomitant synthesis of NIR. The fnrP/nrr double mutant strain lacked both β-galactosidase and the denitrification enzymes, NAR and NIR.

Effect of terminal electron acceptors on FF-lacZ expression

Having established the main responsibility of FnRP for FF site activation, we started to investigate the effects of various

Table 2. Formation of β-galactosidase and denitrification enzymes in the P. denitrificans wild-type and fnrP/nrr mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase (Miller units)</th>
<th>NAR [nkat (mg dry wt)⁻¹]</th>
<th>NIR [nkat (mg dry wt)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pd1222 (wild-type)</td>
<td>1490 ± 50</td>
<td>19.5 ± 0.3</td>
<td>0.41</td>
</tr>
<tr>
<td>Pd29.21 (fnrP)</td>
<td>40.0 ± 0.1</td>
<td>3.2 ± 0.3</td>
<td>0.90</td>
</tr>
<tr>
<td>Pd77.21 (nrr)</td>
<td>343 ± 5</td>
<td>15.0 ± 0.9</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pd92.30 (fnrP/nrr)</td>
<td>9.0 ± 0.1</td>
<td>0.17 ± 0.01</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Table 2. Formation of β-galactosidase and denitrification enzymes in the P. denitrificans wild-type and fnrP/nrr mutant strains

Measurements were made on strains harbouring plasmid pRW2A/FF. Cells were grown oxically and then subjected to micro-oxic conditions for 3 h in succinate medium containing 10 mM nitrate.
terminal acceptors. Strain Pd1222(pRW2A/FF) was therefore grown in succinate medium either oxically or anoxically with a nitrogenous terminal acceptor (nitrate, nitrite or N₂O) until the OD₆₀₀ rose to the required value and then assayed for β-galactosidase activity. As Table 3 indicates, the activity of the melR promoter was strongly influenced not only by oxygen, but, under anoxic conditions, also by the type of nitrogenous electron acceptor present. An exceptionally high level of β-galactosidase was found in the cells grown with N₂O. Interestingly, when N₂O was supplied to the culture growing on nitrate, the resulting β-galactosidase activities were always much lower than for nitrate or N₂O alone.

Parallel activation of the FF-melR promoter and appearance of the membrane-bound NAR

Earlier work by Boublíková et al. (1985) established that some NAR activity is present during micro-oxic incubation of a wild-type strain of *P. denitrificans* even in the absence of added nitrate. Formation of NAR by the Pd1222 (pRW2A/FF) ex-conjugant under such conditions was accompanied by substantial increases in β-galactosidase (Fig. 1). The addition of nitrate had two effects. First, the synthesis of NAR was significantly promoted. Second, the final activity of β-galactosidase was reduced. A plausible explanation for these observations would be that activation by micro-aerobiosis of a transcriptional factor measured with the FF-lacZ fusion (probably FnrP) alone suffices to trigger increased synthesis of NAR. Nitrate seems to be sensed independently, but it or its metabolite may interfere with the oxygen-sensing system to some extent.

The data in Fig. 1 are derived from whole cells and consequently do not discriminate between the membrane-bound and periplasmic NARs. Since these enzymes are known to differ in their sensitivities toward azide (Sears et al., 1993), it was felt worthwhile to examine the dependence of the enzyme activity upon azide concentration. For the cells adapted to micro-oxic conditions without nitrate, a monophasic inhibition curve was obtained corresponding to an I₅₀(N₃⁻) value (the concentration of a given inhibitor which allows an enzyme-catalysed reaction to proceed at 50 % of the uninhibited rate) of 18 μM at 10 mM nitrate (results not shown). This figure agrees well with the kinetic constants Kₘ (nitrate) and Kᵢ (N₃⁻) of 280 μM and 0.55 μM, respectively, reported for the membrane-bound NAR of *P. denitrificans* (Craske & Ferguson, 1986).

### Table 3. Effect of terminal electron acceptors on FF-lacZ expression

<table>
<thead>
<tr>
<th>Terminal acceptor</th>
<th>β-Galactosidase (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂O</td>
<td>659 ± 9</td>
</tr>
<tr>
<td>Nitrate</td>
<td>336 ± 6</td>
</tr>
<tr>
<td>Nitrite</td>
<td>168 ± 2</td>
</tr>
<tr>
<td>O₂</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Nitrate + N₂O</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>Nitrate + nitrite</td>
<td>150 ± 20</td>
</tr>
</tbody>
</table>

In heterologous *E. coli* systems, the NNR- and FnrP-dependent anoxic transcriptions from the FF-melR

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**Fig. 1.** Expression of the FF-lacZ fusion and total NAR upon shift from oxic to micro-oxic conditions. Washed oxically grown cells of *P. denitrificans* Pd1222(pRW2A/FF) were resuspended in succinate medium without (triangles) or with (circles) 10 mM nitrate at 1-2 mg dry wt ml⁻¹ and incubated under limited aeration for up to 3 h. Samples taken at various times were assayed for β-galactosidase (a) and NAR (b). Results of a typical experiment are shown as the means ± SEM; each activity was measured in three to four replicates.
promoter were reported to be activated and inhibited, respectively, by sodium nitroprusside (SNP) as a source of NO$^+$ (Hutchings et al., 2000, 2002). These findings prompted us to investigate which type of response would occur in *P. denitrificans*. Washed oxically grown cells of *P. denitrificans* PD1222(pRW2A/FF) were resuspended in succinate growth medium without nitrate at 1-2 mg dry wt ml$^{-1}$ and incubated with various concentrations of SNP under limited aeration for 1 h. If we regarded the $\beta$-galactosidase activity increments in control samples without SNP (153 Miller units) as 100 %, for 0-001, 0-01, 0-1 and 1 mM SNP the respective relative increments (means ± SEM, n = 3) were 99 ± 4, 83 ± 3, 40 ± 3 and 13 ± 1 %. These results show that SNP significantly inhibits the increase of $\beta$-galactosidase production under oxygen-limiting conditions, indicating a ‘FnrP-type’ response.

**NOR synthesis in response to oxygen withdrawal and to the availability of nitrogenous compounds**

As mentioned in the Introduction, the synthesis of denitrification enzymes observed under micro-oxic conditions could be due to the reduction of trace amounts of nitrate by bacteria, thereby producing the regulatory NO signal. This possibility was minimized in three ways. First, the growth media were pre-incubated with the washed anoxically grown cells to ensure that nitrate concentration was decreased to submicromolar levels where it could not be metabolized easily. Second, we employed a mutant strain devoid of the NO-forming NIR. Third, haemoglobin was included in some experiments as an NO trap. The results obtained using this combined approach are exemplified by Figs 2 and 3. Comparison of panels (a) and (b) in Fig. 2 indicates that a strain containing an NIR mutation behaved like the wild-type strain in its ability to express NOR in response to oxygen limitation without nitrogenous terminal acceptors. The presence of SNP, an NO-generating compound, stimulated a further NOR increase in both strains, at least after 1-5 h treatment, while nitrite was only effective in the wild-type strain. The absence of FnrP influenced neither low-oxygen nor NO response significantly (Fig. 2c). On the contrary, we found no NOR activity in the NNR-deficient strain (results not shown). Haemoglobin (10 $\mu$M) reversed the effects of exogenous NO, but not the effect of hypoxia (Fig. 3). We also asked whether the observed NOR activity does indeed reflect the presence of the respiratory-chain-linked NOR. This could be unequivocally proven by the finding that with succinate as electron donor, the respiratory inhibitor antimycin at a concentration of 3 $\mu$g (mg dry wt)$^{-1}$ reduced the rate of NO consumption to a value below 5 %. Taken together, these results support the idea that both NO-independent and NO-dependent signals are required for the full expression of NOR.

**Activity of the norC promoter**

In an earlier study (van Spanning et al., 1999), expression of NOR was evaluated from the activity of a norC-lacZ promoter fusion in wild-type and the NNR-deficient strains. $\beta$-Galactosidase levels resulting from cultures growing under vigorous shaking were the same in both
Fig. 3. Haemoglobin abolishes the enhancing effect of SNP on NOR expression under micro-oxic conditions. Oxically grown cells of the nirS− strain were harvested and exposed to a limited aeration in the presence of 10 μM SNP (dark grey bars), 10 μM haemoglobin (hatched bars) or a combination of both (light grey bars) for up to 3 h. White bars, control. Results of a typical experiment are shown as the means ± SEM; each activity was measured in three replicates.

strains (96 Miller units). A lower frequency of shaking and a greater volume of culture in the cultivation flasks caused an increase in the wild-type (142 Miller units), while there was a decrease in the nnr mutant (38 Miller units). These experiments were repeated in exactly the same manner with the fnrP mutant. The induction patterns of the norC promoter in this strain (70 and 140 Miller units for the aerated and oxygen-deficient culture, respectively, and a further increase up to 3100 Miller units after adding 100 mM nitrate to the latter) turned out to be similar to the values found in the wild-type cells, adding further evidence for the dispensability of FnRP in NOR expression. It is appropriate to note here that the oxygen limitation conditions originally used in the publication of van Spanning et al. (1999) were not optimized for the production of denitrification enzymes and differ from the micro-oxic incubation protocol applied here. For this reason, we did not attempt to correlate the above-reported norC promoter activities with the enzyme activities of NOR found in other experiments.

DISCUSSION

We conclude that the transcription from the FF-melR promoter in P. denitrificans is mediated chiefly by the FnRP protein based on the following observations. (i) A decrease in promoter activity caused by SNP (this work) resembles a similar result obtained with FnRP in the heterologous E. coli expression system (Hutchings et al., 2002). The reduced sensitivity to SNP inactivation observed by us possibly bears on the fact that the experiments with E. coli were performed under anoxic conditions while the work with P. denitrificans required limited aeration and consequently some of the NO generated from SNP could be trapped by oxygen. (ii) An fnrP knock-out leads to a clear-cut loss of β-galactosidase production (Table 2), showing that the main regulator is encoded by the fnrP gene. However, since the double fnrP/nnr mutant strain manifested somewhat lower β-galactosidase activity than the single fnrP mutant strain, NNR may also have some activation effect, but this is very small in comparison with FnRP. The activity of the FF-melR promoter close to the background value in the double mutant strain indicates that NarR does not activate transcription from this promoter. The possibility that fnrP mutant strains lack active NarR because of regulation of its expression by FnRP is rendered improbable by the results of recent direct measurements of narR promoter activity on the fnrP− background (R. J. M. van Spanning, unpublished data). In Pseudomonas aeruginosa, which possesses proteins ANR and DNR in place of FnRP and NNR, respectively, the FF promoter also became silent as a result of an ann gene mutation (Galimand et al., 1991), whereas a mutation of the dnr gene had no effect (Hasegawa et al., 1998). However, a clear difference with respect to the P. denitrificans system is that the ann mutant strain is devoid not only of ANR, but also of DNR due to the control exerted by ANR over dnr gene transcription (Arai et al., 1997). As a result, its phenotype bears a notable resemblance to that of the fnrP/nnr double mutant of P. denitrificans, i.e. none of the denitrification enzymes is formed. High transcriptional activity of the FF site after transformation with a plasmid expressing the dnr gene indicates that DNR can activate the consensus FNR promoter equally as well as ANR (Hasegawa et al., 1998). Such an overlapping recognition specificity may be less pronounced in P. denitrificans. The low capability of NNR to activate transcription from the FF promoter in P. denitrificans cannot be due to a failure to bind to this region of DNA since the activation takes place in E. coli with the expressed P. denitrificans NNR (Hutchings et al., 2000). A physiological explanation for the different behaviour in both systems is not readily apparent. A challenging, but speculative hypothesis may be that NNR works with an alternative sigma factor in P. denitrificans (cf. the discussion in van Spanning et al., 1997) and that the RNA polymerase containing that sigma factor binds only weakly to the FF promoter.

Although subject to the possible limitations of our plasmid system, the current data argue for the existence of additional signals besides oxygen concentration, capable of modulating FnRP function. One of the factors affecting the activity of FnRP under anoxic conditions may be NO, one of the free intermediates in denitrification. This role of NO is supported by the recently demonstrated sensitivity of the [4Fe–4S] cluster to low concentrations of NO (Wu et al., 2000; Cruz-Ramos et al., 2002), by the observed inactivation of FnRP in vivo by artificial NO donors (Hutchings et al.,
2002; this work), by the retarding effect of nitrate on the transcriptional activation of the FF site under micro-aerobiosis (Fig. 1) and especially by the notable difference between reporter enzyme activities in cells grown with nitrate or nitrite and in cells grown with N₂O (Table 3), which possibly arises from the ability of the former electron acceptors to produce NO. Since the nrr mutation causes nitrite accumulation via reduced expression of NIR (van Spanning et al., 1995) and nitrite in turn inhibits the activity of NOR (Kucéra, 1992), a negative modulation by NO may also underline the lower activity of the FF-melR promoter in the nrr mutant compared to the wild-type strain (Table 1). The mechanism by which nitrogenous terminal acceptors forming NO (nitrate and nitrite) exert their effect can be based on (i) a direct degradation of the [Fe₄S₄] cluster by NO (Cruz-Ramos et al., 2002; Hutchings et al., 2002; Wu et al., 2000) and/or (ii) activation of other transcriptional regulators, which may compete with FnrP for promoter binding, yet only weakly activate expression. Whatever direct or indirect inactivation of FnrP by NO would adversely affect the first reaction of the denitrification pathway, thus providing a negative feedback mechanism that counters the destabilizing positive feedback effect of NO to enhance the expression of NIR and its own production.

Previous measurements of the appearance of enzyme activities, following adaptation of oxically grown batch cultures to the conditions of restricted aeration, have indicated that oxygen limitation alone is the dominant regulatory factor even if nitrate also has some inducing effect on NAR (Kucéra et al., 1984; Boublíková et al., 1985). Our new data lend further support to this original view by demonstrating a partial induction of NAR (Fig. 1) and NOR (Fig. 2) under micro-oxic conditions. Further induction seen upon the addition of nitrogenous compounds suggests the operation of dual control mechanisms involving separate oxygen and nitrogen oxides sensors. For NAR this is in accord with FnrP and NarR acting in concert to regulate the NAR operon (Wood et al., 2001), although some uncertainty persists as to the relative roles of both transcriptional factors. For NOR, the involvement of the NNR-mediated NO signal is well documented, while the molecular basis for the NO-independent part of NOR expression remains unclear at present. One possibility is that a metabolic signal other than NO can also activate NNR-dependent transcription. Physiologically, the dual control of denitrification enzymes possibly reflects a strategy of the cell to get ready for denitrification when oxygen tension becomes limiting.

An alternative approach to study the expression of denitrification enzymes is to measure individual mRNA levels. In this way, results at variance with the dual control model were obtained by Baumann et al. (1996), who failed to detect any induction during the oxic-to-anoxic switch when working with a nitrate-free medium. We think that the discrepancy between our results and those of Baumann et al. (1996) arises essentially from the different concentrations of oxygen in the culture medium used. A number of authors (e.g. Payne et al., 1971; Kučera et al., 1984; Boublíková et al., 1985; Aída et al., 1986) have already demonstrated that the anoxic adaptation of strictly respiring (non-fermenting) bacteria requires a residual respiration of O₂ to produce the metabolic energy inevitable for biosynthesis of the denitrification pathway. This condition was possibly met for our cultures agitated in open flasks but not for Baumann’s chemostat culture sparged with helium. Using an experimental set-up for oxygen limitation similar to ours, Härtig & Zumft (1999) observed a transient accumulation of denitrification gene transcripts in cultures of Pseudomonas stutzeri subjected to a low oxygen tension in the absence of N oxides. Further work is required to characterize such mRNA changes elicited by an appropriately reduced oxygen supply in P. denitrificans.

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References


