Insertion of transposon Tn5 into a structural gene of the membrane-bound nitrate reductase of *Thiosphaera pantotropha* results in anaerobic overexpression of periplasmic nitrate reductase activity

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Chlorate-resistant mutants of the denitrifying bacterium *Thiosphaera pantotropha* were generated by transposon Tn5 mutagenesis. One class was deficient in membrane-bound nitrate reductase activity but retained a periplasmic nitrate reductase activity. Using transposon marker rescue it was shown that in one such mutant, M-6, the transposon was inserted in the membrane-bound nitrate reductase β subunit structural gene (termed *narH* in order to be consistent with the nomenclature of the *Escherichia coli* major nitrate reductase operon). The translated sequence (total of 106 amino acids) from around the point of transposon insertion showed approximately 90% amino acid identity with the β subunits of the *E. coli* nitrate reductases. Under anaerobic growth conditions M-6 overproduced the periplasmic nitrate reductase activity allowing anaerobic growth with nitrate as electron acceptor. A regulatory link was inferred between the presence of the membrane-bound nitrate reductase and expression of the periplasmic nitrate reductase. This is the first demonstration of full denitrification in an organism possessing only a periplasmic nitrate reductase.

Introduction

Thiosphaera pantotropha† is reported to catalyse the complete series of denitrification reactions under aerobic conditions (Robertson & Kuenen, 1983, 1984, 1990; Bell *et al.*, 1990; Bell & Ferguson, 1991; Moir *et al.*, 1993; Berks *et al.*, 1993). The organism has recently been shown to possess two respiratory nitrate reductases, one in the periplasm and the other associated with the cytoplasmic membrane (Bell *et al.*, 1990). The catalytic

site of the membrane-bound enzyme is located on the cytoplasmic face of the cytoplasmic membrane. Of the nitrate reductase activity in cells grown anaerobically with succinate as the carbon source, 80–90% is attributable to the membrane-bound enzyme. Oxygen inhibits reduction of nitrate in bacterial cells expressing the membrane-bound enzyme, apparently by indirectly restricting passage of nitrate across the cytoplasmic membrane (Alefounder & Ferguson, 1980). The periplasmic enzyme accounts for all of the nitrate reductase activity of aerobically grown cells and is active in the presence of oxygen. Activity under aerobic conditions is therefore ascribed to the location of the enzyme in the periplasmic compartment.

Membrane-bound respiratory nitrate reductases have been purified and characterized from several bacteria, most notably and extensively from *Escherichia coli* (Stewart, 1988), but also from the denitrifier *Paracoccus denitrificans* (Craske & Ferguson, 1986; Ballard & Ferguson, 1987, 1988). In contrast, the occurrence of periplasmic nitrate reductases is much less widely documented and it is only for *Rhodobacter sphaeroides* f.sp. *denitrificans*, which also expresses a membrane-bound nitrate reductase (Byrne & Nicholas, 1987), and some strains of *Rhodobacter capsulatus* that character-

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Abbreviations: BV⁺, reduced benzyl viologen; MV⁺, reduced methyl viologen.

The EMBL accession number for the sequence reported in this paper is Z26255.

† Ludwig *et al.* (1993) have recently suggested, principally on the basis of 16S rRNA sequences, that *T. pantotropha* should be reclassified as *Paracoccus denitrificans*. While we agree that the organisms are very closely related, aspects of the biochemistry are distinct (unpublished data; manuscripts in preparation). Thus we think that reclassification is premature. We therefore continue to refer to the strain as *T. pantotropha* pending clarification by the International Committee of Systematic Bacteriology.

ization has been achieved to any significant extent (Alef & Klemme, 1979; Satoh, 1981; McEwan *et al.*, 1987; Richardson *et al.*, 1990). The periplasmic nitrate reductases from *R. capsulatus* and *T. pantotropha* are similar with respect to two catalytic properties that distinguish them from the membrane-bound enzyme; resistance to inhibition by azide and inability to reduce chlorate (Bell *et al.*, 1990; Ferguson *et al.*, 1987).

The expression of two distinct respiratory nitrate reductases in *T. pantotropha* raises questions as to the mechanism of their differential regulation. It has recently been reported that expression of the periplasmic enzyme is higher following aerobic growth on reduced carbon substrates, such as butyrate or caproate, than on more oxidized substrates, such as malate or succinate (Richardson & Ferguson, 1992). Further, the presence or absence of nitrate had no effect on aerobic expression of the periplasmic enzyme. Under anaerobic growth conditions it was found that nitrate reductase activity is predominantly due to the membrane-bound enzyme and is not significantly affected by the carbon growth substrate. Expression of the periplasmic enzyme thus remains low under anaerobic conditions regardless of the growth substrate. This is not true of the periplasmic nitrate reductase of *R. capsulatus* N22DNAR⁺ which is expressed to high levels during anaerobic phototrophic growth, the enzyme being further induced when highly reduced carbon sources are used for growth or if nitrate is available as an auxiliary electron acceptor (Richardson *et al.*, 1990). Aerobic growth results in decreased expression of the periplasmic enzyme (D. J. Richardson & S. J. Ferguson, unpublished results). Thus despite having similar catalytic properties the periplasmic nitrate reductases of *T. pantotropha* and *R. capsulatus* have distinct patterns of regulation. We reasoned that the differential regulation of the periplasmic nitrate reductase in *T. pantotropha* and *R. capsulatus* in response to oxygen might be a consequence in part of the capacity of *T. pantotropha*, but not *R. capsulatus*, to express a membrane-bound nitrate reductase. To investigate this theory we have isolated mutants of *T. pantotropha* deficient in the membrane-bound nitrate reductase and have examined the resultant effect on expression of the periplasmic nitrate reductase.

Methods

Thiosphaera pantotropha (strain LMD 82.5) was obtained from Dr L. A. Robertson, Department of Microbiology, Technical University of Delft, Delft, The Netherlands. Cells were cultured at 37 °C on the minimal salts medium described by Robertson & Kuenen (1984). The carbon source was 30 mM-succinate and the concentration of nitrate when present was 25 mM for anaerobic growth and 5 mM for aerobic growth. The organism was grown aerobically in 50 ml cultures in 300 ml conical flasks. For anaerobic growth cultures were inoculated into 28 ml Universal bottles containing 26 ml medium and immediately

sparged with nitrogen gas. Cells were harvested by centrifugation at mid-exponential phase ($OD_{650} = 0.6$, equivalent to 0.3–0.4 mg dry wt cells ml⁻¹). At this stage of growth the oxygen concentration in the aerobic cultures (measured *in situ* using a Jenway 9070 portable oxygen meter) was 180 to 200 µM. Cells were resuspended in nitrate-free growth medium for activity assays. Cells were fractionated as described by Bell *et al.* (1990). Nitrate respiration was measured under anaerobic and aerobic conditions using a nitrate electrode as previously described (Bell *et al.*, 1990). Methyl- and benzyl-viologen-linked nitrate reductase assays were as described by Bell *et al.* (1990).

Transposon Tn5 mutagenesis was carried out according to the method of Chandra & Friedrich (1986). Megaplasmid analysis was by the method of Eckhardt (1978). General cloning techniques were as described by Sambrook *et al.* (1989). DNA sequencing was performed using both Sequenase version 2.0 with [³⁵S]dATP and the automated Pharmacia laser fluorescent system (A.L.F. Sequencer). The primer used was 5'-CGTTCAGGACGCTAC-3', complementary to bases 17–34 within the Tn5 inverted repeat (Auerswald *et al.*, 1980). Sequence analysis made use of the University of Wisconsin Genetics Computing Group software package release 7.2 (Devereux *et al.*, 1984).

Results

Selection and characterization of chlorate-resistant Tn5-insertion mutants of T. pantotropha

The membrane-bound nitrate reductase of *T. pantotropha*, but not the periplasmic enzyme, will reduce chlorate to the toxic chlorite (Bell *et al.*, 1990). Both aerobically and anaerobically grown *T. pantotropha* cells possess nitrate reductase activity, but tests of sensitivity to chlorate established that in cells grown aerobically in liquid culture there was resistance to 300 mM chlorate. In contrast, under anaerobic conditions growth was strongly retarded by 10 mM-chlorate. Under the latter conditions the membrane-bound rather than the periplasmic enzyme is predominantly expressed, consistent with induction of the membrane-bound nitrate reductase being responsible for the sensitivity towards chlorate.

Growth on agar plates was also sensitive to 10 mM-chlorate under anaerobic conditions. However, under aerobic conditions the sensitivity to chlorate increased relative to liquid cultures. Bacterial colonies growing on agar plates incubated aerobically experience low oxygen tensions and localized anaerobiosis. In *T. pantotropha* this results in low-level induction of the membrane-bound nitrate reductase (ascertained by picking colonies off plates, resuspending in phosphate buffer and determining the relative benzyl- and methyl-viologen-dependent nitrate reductase activities as described by Bell *et al.*, 1990). Consequently, during aerobic growth on plates, although resistance was still observed to concentrations of chlorate of up to 100 mM, increasing the chlorate concentration to 300 mM strongly inhibited growth.

At the outset of this work it was not clear whether mutants deficient in the membrane-bound nitrate reductase would be able to grow with nitrate under

anaerobic conditions because of the recognized failure of wild-type cells to express the periplasmic enzyme to high levels under these growth conditions (Bell *et al.*, 1990; Richardson & Ferguson, 1992). On agar plates incubated aerobically a mutant deficient in membrane-bound nitrate reductase would still utilize oxygen for growth but would not produce chlorite from chlorate in the portion of the colony experiencing microanaerobiosis. It was therefore decided to seek chlorate-resistant mutants by plating transposon Tn5 transconjugants on plates containing 300 mM chlorate and to incubate these aerobically.

Several classes of chlorate-resistant Tn5-insertion mutants were isolated. The phenotype of one class, comprising three isolates obtained from 3000 colonies, will be described. Detailed presentation will be restricted to a representative mutant, strain M-6. Under aerobic conditions with succinate as carbon source growth of this class of mutants was similar to that of the wild-type cells (data for M-6 growth in liquid culture are given in Table 1). The mutants also grew under anaerobic conditions with succinate as carbon source and nitrate as electron acceptor though at a rate that was threefold lower than the wild-type (Table 1). This growth rate was unchanged by the presence of 50 mM-chlorate (Table 1). In contrast, anaerobic growth of the wild-type cells was completely inhibited by 50 mM-chlorate.

Anaerobic growth of the mutants, like growth of the wild-type, resulted in the evolution of gas suggesting that nitrate was still fully reduced to molecular dinitrogen by the mutants (the solubility of nitrous oxide is sufficiently high that it is very improbable that this was the major gaseous product). Anaerobic growth with nitrite or nitrous oxide as added electron acceptors was identical for the wild-type and mutant cells indicating that the mutants had no pleiotropic deficiency in the denitrification pathways terminating in nitrite or nitrous oxide reductases. The mutants grew indistinguishably from the wild-type cells; (i) chemoautotrophically with thio-sulphate and bicarbonate; (ii) chemoautotrophically with formate as the sole carbon source; (iii) with xanthine as sole carbon and nitrogen source. These experiments indicate that there was no lesion in biosynthesis of the molybdopterin cofactor thought to be common to the nitrate reductases (Rajagopalan & Johnson, 1992), most formate dehydrogenases (Harms *et al.*, 1985), at least one necessary reaction of thiosulphate oxidation in *T. pantotropha* (Chandra & Friedrich, 1986; Friedrich *et al.*, 1986) and also some bacterial xanthine dehydrogenases (Hetttrich *et al.*, 1991; Johnson *et al.*, 1991). Megaplasmid analysis of the mutant cells confirmed that the mutants were (a) *T. pantotropha* and (b) had the full complement of megaplasmids found in the wild-type cells (Chandra & Friedrich, 1986).

Table 1. Comparison of anaerobic and aerobic growth rates of wild-type *T. pantotropha* and the chlorate-resistant mutant M-6

The carbon source was succinate. Growth was followed by measuring the increase in light scattering at 650 nm.

Strain	Doubling time (min)		
	Growth conditions... 25 mM-NO ₃ ⁻ + 50 mM-ClO ₃ ⁻	Anaerobic 25 mM-NO ₃ ⁻	Aerobic 5 mM-NO ₃ ⁻
Wild-type	No growth	80	55
M-6	240	240	55

Table 2. Sensitivity to azide of rates of aerobic and anaerobic nitrate reduction in cells of wild-type *T. pantotropha* and mutant M-6 grown under aerobic and under anaerobic conditions

The steady-state oxygen concentration was 200 µM in the aerobic experiments. Cells were resuspended in fresh nitrate-free growth medium.

Assay conditions	Rate of nitrate reduction [nmol nitrate reduced min ⁻¹ (mg cell protein) ⁻¹]			
	Wild-type		M-6	
	After anaerobic growth	After aerobic growth	After anaerobic growth	After aerobic growth
Anaerobic	900	130	950	135
Anaerobic + 20 µM-azide	70	115	850	130
Aerobic	90	95	750	100
Aerobic + 20 µM-azide	90	95	725	95

The anaerobic growth, coupled with the resistance to chlorate, suggested that this class of chlorate-resistant mutant had retained the periplasmic nitrate reductase but lost the membrane-bound nitrate reductase. To investigate this possibility the properties of nitrate respiration by intact M-6 cells, grown anaerobically with succinate as carbon source and nitrate as electron acceptor were examined (Table 2). In the wild-type cells nitrate respiration was strongly inhibited by azide and oxygen, consistent with expression of the membrane-bound enzyme (Bell *et al.*, 1990). In mutant M-6 the rate of nitrate respiration was comparable to that of the wild-type despite the much lower growth rate (Table 1). However, in contrast to the wild-type, this rate was not strongly depressed either by azide or oxygen. This is characteristic of the periplasmic enzyme and suggests that M-6 overexpresses this enzyme activity during

Table 3. Comparison of the nitrate reductase activities of intact cells of wild-type and mutant M-6 cells of *T. pantotropha* grown with succinate as carbon source using reduced methyl or benzyl viologen as electron donor

Cells were resuspended in 100 mM-HEPES/NaOH, pH 7.3.

Growth conditions	Activity [nmol viologen oxidized min ⁻¹ (mg cell protein) ⁻¹]	
	MV ⁺	BV ⁺
Wild-type		
Anaerobic	30	215
Aerobic	60	55
Mutant M-6		
Anaerobic	235	240
Aerobic	70	60

Table 4. Subcellular localisation of nitrate reductase activities from anaerobically grown wild-type *T. pantotropha* and mutant M-6

The total activity (sum of all fractions) was around 2700 nmol MV⁺ oxidized min⁻¹ in both cases. This represented the total activity recovered from 30 mg dry wt of anaerobically grown cells.

Strain	Percentage MV ⁺ -nitrate oxidoreductase activity recovered in:		
	Periplasm	Membranes	Cytoplasm
Wild-type	15	85	1
Mutant M-6	100	0	0

anaerobic growth. The rates of nitrate respiration in aerobically grown wild-type and mutant M-6 cells were comparable but were sevenfold lower than in anaerobically grown cells. The insensitivity to oxygen and azide indicated that the activity was attributable to the periplasmic nitrate reductase in both cases.

In intact cells the membrane-bound nitrate reductase is more active with reduced benzyl viologen (BV⁺) than reduced methyl viologen (MV⁺) as electron donor, while the periplasmic nitrate reductase does not discriminate between the two (Bell *et al.*, 1990). This is because MV⁺ is relatively ineffective at crossing the cytoplasmic membrane to interact with the catalytic subunit of the membrane-bound nitrate reductase (Jones & Garland, 1977). As a consequence, in wild-type anaerobically grown cells the rate of BV⁺-dependent nitrate reduction was much greater than the MV⁺-dependent rate (Table 3). However, in anaerobically grown mutant M-6 the nitrate reductase activity was similar with either BV⁺ or MV⁺ as the non-physiological reductant. As the reduced viologens are direct electron donors to the nitrate reductases these experiments suggest that M-6 expresses

high levels of the periplasmic nitrate reductase rather than the membrane-bound nitrate reductase when grown anaerobically. Both the wild-type and mutant M-6 showed similar levels of periplasmic nitrate reductase activity when grown aerobically with succinate as the carbon source (Table 3; see also Table 2 results). With both strains aerobic growth on the more reduced carbon source butyrate led to similar increased levels of periplasmic nitrate reductase activity (data not shown), in agreement with results previously reported for the wild-type (Richardson & Ferguson, 1992). These experiments show that the pattern of expression of the periplasmic nitrate reductase activity during aerobic growth is not affected in mutant M-6, i.e. expression of the periplasmic nitrate reductase activity in mutant M-6 is not constitutively derepressed.

Studies on subcellular fractions of mutant M-6

Wild-type *T. pantotropha* and mutant M-6, each grown anaerobically with nitrate as electron acceptor, were fractionated into periplasmic, cytoplasmic and membrane fractions (Table 4). Consistent with previous observations (Bell *et al.*, 1990), 80% of the nitrate reductase activity of the wild-type cells was associated with the membrane fraction. In contrast, no nitrate reductase activity was detected with the membranes from mutant M-6. Instead the activity was associated exclusively with the periplasmic fraction and was sevenfold greater than that observed in wild-type periplasmic fractions.

Dithionite-reduced minus nitrate-oxidized spectra of membranes from anaerobically grown wild-type cells of *T. pantotropha* show reoxidation by nitrate of cytochrome(s) with absorption maxima at 557 nm, characteristic of *b*-type cytochrome (Fig. 1*c*). By analogy with the membrane-bound nitrate reductases of *E. coli* and *P. denitrificans* (Sodergren *et al.*, 1988; Ballard & Ferguson, 1988) this nitrate-oxidizable cytochrome *b* is probably associated with the integral membrane γ -subunit. Absence of an absorption change at 550 nm suggests that there is no oxidation of membrane *c*-type cytochromes by nitrate. The dithionite-reduced spectrum of membranes from anaerobically grown mutant M-6 was rather similar to that of the wild-type (compare Fig. 1*d* with Fig. 1*a*). In contrast, no nitrate-dependent oxidation of either *b*- or *c*-type cytochrome was evident for the M-6 membranes (Fig. 1*e, f*). These spectroscopic studies are consistent with the absence of membrane-bound nitrate reductase in mutant M-6.

Taken together, these experiments on subcellular fractions confirm the view derived from the whole cell experiments that under anaerobic growth conditions mutant M-6 overexpresses the periplasmic nitrate

Fig. 1. Oxidation of membrane cytochromes by nitrate in wild-type *T. pantotropha* but not in mutant M-6, each grown anaerobically. Membranes were suspended in 100 mM-Tris/HCl, pH 7.8, at a concentration of 2.5 mg protein ml⁻¹ (a-c) or 1.0 mg protein ml⁻¹ (d-f). (a) Wild-type membranes reduced with sodium dithionite; (b) sample from (a) after addition of 1 mM-potassium nitrate; (c) the difference spectrum obtained by subtracting the spectrum in (b) from that in (a), allowing the component(s) oxidized by nitrate to be identified; (d) dithionite reduced membranes from mutant M-6; (e) following addition of 1 mM-nitrate to the sample shown in (d); (f) the difference spectrum obtained by subtracting the spectrum in (e) from that in (d). Spectra were recorded at room temperature using an Aminco DW 2000 spectrophotometer.

reductase activity and does not express a functional membrane-bound nitrate reductase.

Identification of the locus of transposon insertion in mutant M-6

Southern blotting of mutant M-6 chromosomal DNA with the plasmid pSUP202::Tn5 (Simon *et al.*, 1983) that was used to generate the transposon mutants

established the presence of a single integrated transposon, while Southern blotting with pSUP202 alone indicated the absence of vector DNA co-integration at the site of Tn5 insertion. *T. pantotropha* chromosomal DNA flanking the integrated transposon was cloned in pUC18 as a 13.5 kb *Eco*RI restriction fragment from total M-6 DNA using the transposon-encoded kanamycin resistance as a selectable marker. The size of the cloned insert corresponded to the size of the Tn5-

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-149  ACCTTCATGATGATCTGCCGCGGCTGTGCGAGCATTGCCTCAACCGGCCTGC  -96
      ThrPheMetMetTyrLeuProArgLeuCysGluHisCysLeuAsnProAlaCys

-95   GTTGCCTCCTGCCCCCTCGGGCGCGATCTACAAGCGGAAGAGGACGGGGTGGTG  -42
      ValAlaSerCysProSerGlyAlaIleTyrLysArgGluGluAspGlyValVal

-41   CTGATCGACCAGGACAAGTGCCTGGGGCTGGCGGATGTGTCTCGGGCTGCCCC  12
      LeuIleAspGlnAspLysCysArgGlyTrpArgMetCysValSerGlyCysPro
      *****

13    TACAAGAAGATCTATTACAACCTGGTCGACCGGGAAGTCGAAAAATGCATCCTG  66
      TyrLysLysIleTyrTyrAsnTrpSerThrGlyLysSerGluLysCysIleLeu

67    TGCTATCCGCGCCTTGAATCGGGCCAGCCACGGTCTGCTCGGAAACCTGCGTC  120
      CysTyrProArgLeuGluSerGlyGlnProThrValCysSerGluThrCysVal

121   GGGCGCATCCGCTACCTGGGCGTGATGCTTTACGATGCCGACAAGATC      168
      GlyArgIleArgTyrLeuGlyValMetLeuTyrAspAlaAspLysIle

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Fig. 2. DNA sequence and deduced amino acid sequence around the point of transposon Tn5 insertion in mutant M-6. The 9 base pairs duplicated at the point of transposon insertion are marked by asterisks; position zero in the DNA sequence is arbitrarily assigned to the central base of this duplicated sequence.

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M-6      TFMMYLPR LCEHCLNPAC VASCPGAIY KREEDGVVLI DQDK
NarH (175-216) TFMMYLPR LCEHCLNPAC VATCPGAIY KREEDGVILI DQDK
NarY (174-215) TFMMYLPR LCEHCLNPSC VTTCPGAIY KREEDGVILI DQDK
NorB (179-220) TVFFYLPR ICNHCLNPGC VAACPQAIY KRGEDGVVLV SQER

Orf3 (42-81)   TSTITAPV VCHHCEAPC LQVCPVNAIS Q..RQDAIQL NESL
HycB (42-81)   NEKESAPQ LCHHCEAPC AVVCPVNAIT R..VDGAVQL NESL
CooF (56-95)   VDGATAMP QCRQCEDAPC TFACPTGACR Q..ADGQVQI VEQH
DmsB (58-98)   VFAYYLSI SCNHCEAPC TKVCPSGAMH KR.EDGFVVV DEDV
PsrB (52-92)   LSPNYHRQ SCVQCENTPC VSVCPTKASY VN.EDGIVSV NVDL
FdnH (91-132) LEWLIRKD GCIDCEDPGC LKACPSAGAI IQYANGIVDF QSEN
FdhB (50-90)   GKEKSLSI ACMHCSAPC AQCVPVDCFY VR.ADGIVLIH DKEK
      * * * * *
      -----2-----

M-6      CRGWRM CVSGCPYKKI YYNWST.....GKSEKICLCYP.
NarH (217-249) CRGWRM CITGCPYKKI YFNWKS.....GKSEKICFCYP.
NarY (216-248) CRGWRL CISGCPYKKI YFNWKS.....GKSEKICFCYP.
NorB (221-253) CRAWRM CVSGCPYKKT YFNWST.....GKAKEKICLCYP.

Orf3 (82-151)   CIGCKL CAVVCPFGAI SASGSR. (37aa) .TVAVKCDLCDF.
HycB (82-148)   CVSCKL CGIACPFPGAI EFSGSR. (34aa) .AIAVKCDLCDF.
CooF (96-139)   CIGCKL CVMVCPFGAI TVRSET. (10aa) .GVAKKCDLCVDW
DmsB (99-131)   CIGCRY CHMACPYGAP QVNETK.....GHMTKCDGCVYD.
PsrB (93-126)   CVGCLY CIAACPYPQAR YVDPVT.....KAPDKCNFCKDT
FdnH (133-165) CIGCGY CIAGCPFPNP RLKED.....NRVYKCTLCDV.
FdhB (91-129)   CIGCGY CLYACPFPGAP QFPKSG.. (5aa) .GPMDKCTFCAGG
      * * * * *
      -----3-----

M-6      .....RL.ESGQPTVCSETCV GRIRYLGVL YDADKI
NarH (250-280) .....RI.EAGQPTVCSETCV GRIRYLGVL YDADAI
NarY (249-279) .....RI.ESGQPTVCSETCV GRIRYLGVL YDADRI
NorB (254-284) .....RL.ESGHAPACFHSCV GRIRYIGLVL YDADAI

Orf3 (152-179) .....LPEGPACVRACP NQALRLITGD SLQRQM
HycB (149-176) .....DEQGPACARMCP TKALHLVDNT DIARVS
CooF (140-171) .....PASTGKTAPACVEACP TKAIRMVLDL AYRIAL
DmsB (132-162) .....RV.AEGKKPICVESCP LRALDFGPID ELRKKH
PsrB (127-157) .....RL.ARGEEPACVTVCP TDALTFGDMS DPKSKI
FdnH (166-196) .....RV.SVGQEPACVKTCP TGAHFCTGK EMLELA
FdhB (130-176) .....RI.AEGKVPVCAAMCS TKALLAGDSD SISLII
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Fig. 3. Comparison of the protein sequence derived from the nucleic acid sequence around the point of transposon Tn5 insertion in mutant M-6 with the amino acid sequences of the iron-sulphur protein subunits of the membrane-bound molybdopterin dinucleotide and other enzymes. Asterisks designate cysteines that are postulated to be involved in liganding iron-sulphur centres. Shading indicates residues that are identical between the M-6 and other sequences. The sequences are: NarH, *E. coli* membrane-bound nitrate reductase A (Blasco *et al.*, 1989); NarY, *E. coli* membrane-bound nitrate reductase Z (Blasco *et al.*, 1990); NorB, *Nitrobacter hamburgensis* nitrite oxidase (Kirsten & Bock, 1992); Orf3, *E. coli* (Andrews *et al.*, 1991); HycB, *E. coli* formate hydrogenlyase operon (Böhm *et al.*, 1990); CooF, *Rhodospirillum rubrum* carbon monoxide dehydrogenase (Kerby *et al.*, 1992); DmsB, *E. coli* dimethylsulphoxide reductase (Bilous *et al.*, 1988); PsrB, *Wolinella succinogenes* polysulphide reductase (Krafft *et al.*, 1992); FdnH, *E. coli* nitrate-inducible formate dehydrogenase (Berg *et al.*, 1991); FdhB, *W. succinogenes* formate dehydrogenase (Bokranz *et al.*, 1991).

hybridizing *Eco*RI fragment detected in the Southern analysis. For sequencing, M-6 chromosomal DNA flanking the 5' end of the Tn5 element was subcloned as

a 4.7 kb *Bam*HI fragment containing 1.7 kb of chromosomal DNA, and M-6 DNA flanking the Tn5 3' end was subcloned as a 4 kb *Sal*I-*Eco*RI fragment containing

approximately 1 kb of chromosomal DNA. M-6 DNA immediately adjacent to the transposon ends was then sequenced using a primer complementary to sequence within the Tn5 inverted repeat. The sequence, obtained independently by both manual and automated sequencing, around the point of insertion is shown in Fig. 2. The expected 9 bp duplication with GC base pairs at positions 1 and 9 (Berg *et al.*, 1983) is observed at the point of Tn5 integration. As shown in Fig. 3, translation of one of the reading frames around the point of insertion shows strong sequence similarity to the iron-sulphur protein (β) subunits of membrane-bound molybdopterin dinucleotide enzymes, including those of the membrane-bound nitrate reductases of *E. coli* and the nitrite oxidase of *Nitrobacter hamburgensis*, for which a close structural relationship to the membrane-bound nitrate reductases has been expected (Ingledew & Halling, 1976; Ballard & Ferguson, 1987, 1988). It is clear from Fig. 3 that the similarity is greatest with the nitrate reductases/nitrite oxidase; 91%, 90%, 75% amino acid identity and 98%, 98%, 89% amino acid similarity to NarH, NarY, NorB respectively. Additionally, the pattern of insertions and deletions in the M-6 sequence relative to the other iron-sulphur proteins is the same as that found in the nitrate reductases (Fig. 3). Finally Trp₂₂₀ (*E. coli* NarH numbering), the functional significance of which is discussed below and which is found only in the nitrate reductases/nitrite oxidase, is conserved in the M-6 sequence. We conclude from this sequence analysis that the transposon insertion in mutant M-6 is in the structural gene for the β -subunit of the membrane-bound nitrate reductase.

Discussion

The results of this study confirm that in wild-type cells of anaerobically grown *T. pantotropha* nitrate reductase activity is predominantly attributable to the membrane-bound enzyme. However, chlorate-resistant transposon Tn5-insertion mutants that do not express an active membrane-bound nitrate reductase retain wild-type levels of nitrate reductase when grown anaerobically, this activity being entirely attributable to the periplasmic nitrate reductase. As a result these mutants have a much higher capacity for aerobic nitrate respiration after anaerobic growth than do wild-type cells grown under identical conditions (Table 2).

In three-subunit membrane-bound molybdopterin dinucleotide enzymes, including the *E. coli* nitrate reductases, the genes coding for the three structural subunits are transcribed in the order molybdopterin dinucleotide-containing catalytic (α) subunit (*narG* in the major *E. coli* nitrate reductase), iron-sulphur-centre-containing (β) subunit (*narH*) and quinol/quinone-binding membrane-

anchored (γ) subunit (*narI*; for references see Fig. 3 legend). In mutant M-6 it was demonstrated by sequencing across the point of transposon insertion that the insert is in the structural gene for the β -subunit of the membrane-bound nitrate reductase. Given that the point of insertion corresponds to a position approximately in the middle of the β polypeptides of the two nitrate reductases in *E. coli*, it is unlikely that a functional β subunit is produced in mutant M-6. If the organization of the structural genes for the subunits of the membrane-bound nitrate reductase of *T. pantotropha* is the same as that found in all previously described membrane-bound molybdopterin dinucleotide enzymes, then the gene for the γ subunit will be downstream of the point of transposon insertion in mutant M-6. As transposon Tn5 usually blocks the transcription of genes distal from the operon promoter (Berg, 1989) it is unlikely that any expression of the γ polypeptide occurs. Thus we expect that only the α subunit of the membrane-bound nitrate reductase would be synthesized intact in this mutant. This is consistent with the observed phenotype of M-6: lack of membrane-bound nitrate reductase activity and an inability to link reduction of chlorate to physiological electron donors. Similarly, we can rationalize the observations of lack of nitrate-oxidizable *b*-type cytochrome in membranes from anaerobically grown cells of the mutant as being caused by the loss of transcription of the *b*-haem-binding γ -subunit structural gene. No reduced-viologen-dependent nitrate reductase activity was found in the cytoplasmic or membrane fractions of anaerobically grown mutant M-6 suggesting that if the α -subunit is still synthesized in this mutant then either (i) reduced viologens cannot donate electrons directly to the α subunit, or (ii) the molybdopterin dinucleotide cofactor cannot be properly inserted in the isolated α -subunit, or (iii) the isolated α -subunit is susceptible to proteolysis or other inactivation *in vivo*.

The β subunits of the membrane-bound molybdopterin-dinucleotide-containing enzymes contain four clusters of cysteine residues that are proposed to provide ligands to four $[4\text{Fe-4S}]^{2+,1+}$ centres, or in the case of the nitrate reductases and nitrite oxidase three $[4\text{Fe-4S}]^{2+,1+}$ and one $[3\text{Fe-4S}]^{1+,0}$ centres (reviewed in Weiner *et al.*, 1992). Although in defining the point of transposon insertion in mutant M-6 we have sequenced only a portion of the β subunit this region covers three of the four cysteine clusters (see Fig. 3). All eleven cysteines conserved in this region in the membrane-bound nitrate reductases/nitrite oxidase are also found in the *T. pantotropha* sequence (Fig. 3). As each motif of four clustered cysteine residues is believed to provide three ligands for one iron-sulphur centre and one ligand for a second centre we deduce that four iron-sulphur centres are present in the β subunit of *T. pantotropha*. The

substitution of a tryptophan for a cysteine at position 220 (*E. coli* NarH numbering) in the nitrate reductase/nitrite oxidase sequences relative to the other molybdopterin enzyme iron-sulphur protein sequences is probably associated with the presence of a $[3\text{Fe-4S}]^{1+0}$ centre in these enzymes (Johnson *et al.*, 1985; Rothery & Weiner, 1991; Guigliarelli *et al.*, 1992; Meincke *et al.*, 1992; note that the converse substitution does not necessarily lead to a $[4\text{Fe-4S}]^{2+1+}$ centre; Augier *et al.*, 1993). The tryptophan-for-cysteine substitution is also apparent in the *T. pantotropha* β subunit and so we predict that the *T. pantotropha* membrane-bound nitrate reductase will contain a $[3\text{Fe-4S}]^{1+0}$ centre.

The sequence reported here is the first from any part of the membrane-bound nitrate reductase of a denitrifying bacterium. The very high sequence similarity between the *T. pantotropha* nitrate reductase partial sequence and the *E. coli* nitrate reductase sequences suggests that the membrane-bound nitrate reductases of denitrifying bacteria will turn out to be structurally very similar to those of the non-denitrifying enteric bacteria. Such a relationship had been indicated previously by similar subunit structure and antibody cross-reactivities (Craske & Ferguson, 1986; Ballard & Ferguson, 1988; Ballard *et al.*, 1990). That insertion of a single transposon abolished all membrane-bound nitrate reductase activity in mutant M-6 means that, under the conditions investigated, *T. pantotropha*, unlike *E. coli* (Iobbi *et al.*, 1987; Blasco *et al.*, 1990), expresses only a single active membrane-bound nitrate reductase. Southern analysis with a fragment of the membrane-bound nitrate reductase operon derived from the 13.5 kb *EcoRI* M-6 fragment shows that there is only a single copy of the operon and is an indication against the possibility of a cryptic gene duplication (F. Outen, M. D. Page & B. C. Berks, unpublished results).

T. pantotropha mutant M-6 is the first organism possessing only a periplasmic nitrate reductase that has been shown to denitrify. If the periplasmic nitrate reductase activity provided identical stoichiometry of proton translocation across the plasma membrane as the membrane-bound enzyme, then one might reasonably expect identical growth rates and growth yields in the mutant and wild-type unless the rate of nitrate reduction provided by the periplasmic enzyme were to be significantly less than that provided by the membrane-bound enzyme. The latter is demonstrated not to be the case here. Thus lower growth rates, and yields during denitrification under certain conditions (D. J. Richardson, unpublished observations), of mutant M-6 compared with the wild-type are plausibly a consequence of reduced energy conservation by the periplasmic nitrate reductase relative to the membrane-bound nitrate reductase in the span ubiquinol to nitrate. Such a difference

in energetic coupling is in accord with current thinking. Quinol oxidation by the membrane-bound nitrate reductase generates a protonmotive force with a probable proton translocation stoichiometry of $2\text{H}^+:2\text{e}^-$ (Jones *et al.*, 1980; Parsonage & Ferguson, 1983; Nicholls & Ferguson, 1992). In contrast, quinol oxidation by the periplasmic enzyme is postulated not to generate a protonmotive force since oxidation of quinol results in release of the two electrons, and presumably the two protons, in the periplasm, all of which are then consumed in the nitrate reduction reaction. This lack of energy conservation is consistent with the proposed role of the periplasmic nitrate reductase as an electron sink (McEwan *et al.*, 1984; Richardson *et al.*, 1988; Richardson & Ferguson, 1992).

Mapping of the mutation in mutant M-6 to the structural gene for the membrane-bound nitrate reductase suggests that overexpression of the periplasmic nitrate reductase activity during anaerobic growth is a consequence of the loss of the membrane-bound nitrate reductase activity and rules out mutation in a regulatory protein as a cause. The elevated levels of periplasmic nitrate reductase activity in anaerobically grown mutant M-6 are similar to the levels of activity of the enzyme in wild-type cells grown aerobically on highly reduced carbon substrates (Richardson & Ferguson, 1992). Failure to express the membrane-bound nitrate reductase (a quinol oxidase) could lead to a decrease in the steady-state redox potential of the quinol/quinone pool. A plausible explanation for the elevated expression of the periplasmic nitrate reductase in mutant M-6 is thus that it is mediated by a regulatory system that responds to changes in the redox state of the cell.

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