The NapF protein of the *Escherichia coli* periplasmic nitrate reductase system: demonstration of a cytoplasmic location and interaction with the catalytic subunit, NapA

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The periplasmic nitrate reductase of *Escherichia coli* is important during anaerobic growth in low-nitrate environments. The *nap* operon encoding this nitrate reductase comprises seven genes including a gene, *napF*, that encodes a putative cytoplasmic iron–sulphur protein of uncertain subcellular location and function. In this study, N-terminal sequence analysis, cell fractionation coupled with immunoblotting and construction of LacZ and PhoA fusion proteins were used together to establish that NapF is located in the *E. coli* cytoplasm. A bacterial two-hybrid protein–protein interaction system was used to demonstrate that NapF interacted in the cytoplasm with the terminal oxidoreductase NapA, but that it did not self-associate or interact with other electron-transport components of the Nap system, NapC, NapG or NapH, or with another cytoplasmic component, NapD. NapF, purified as a His₆-tagged protein, exhibited spectral properties characteristic of an iron–sulphur protein. This protein was able to pull down NapA from soluble extracts of *E. coli*. A growth-based assay for NapF function in intact cell cultures was developed and applied to assess the effect of mutation of a number of conserved amino acids. It emerged that neither a highly conserved N-terminal double-arginine motif, nor a conserved proline motif, is essential for NapF-dependent growth. The combined data indicate that NapF plays one or more currently unidentified roles in the post-translational modification of NapA prior to the export of folded NapA via the twin-arginine translocation pathway into the periplasm.

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INTRODUCTION

The *Escherichia coli* genome encodes three distinct nitrate reductases. The membrane-bound nitrate reductases NarA and NarZ reduce nitrate in the cytoplasm (Berks *et al.*, 1995a; Blasco *et al.*, 1990; Iobbi-Nivol *et al.*, 1990; Stewart, 1988). Nitrate reductase A is the predominant nitrate reductase when *E. coli* is grown under nitrate-sufficient conditions (Potter *et al.*, 1999), whereas the poorly expressed nitrate reductase Z confers a selective advantage during the stationary phase or very slow growth (Iobbi *et al.*, 1987, 1990; Clegg *et al.*, 2006). Under nitrate-limiting conditions, a periplasmic nitrate reductase (Nap) is expressed (Potter *et al.*, 1999; Wang *et al.*, 1999; Stewart *et al.*, 2002). Periplasmic nitrate reductases have been found in many different organisms, where they fulfil different physiological roles, depending on the species. For example, they can function as electron sinks during photosynthesis in

Rhodobacter species (Reyes *et al.*, 1996, 1998) and during aerobic growth of *Paracoccus* species on highly reduced carbon sources (Richardson & Ferguson, 1992; Sears *et al.*, 2000), or they may be used during anaerobic respiration, as in *E. coli* (Potter & Cole, 1999; Brondijk *et al.*, 2002; Stewart *et al.*, 2002). In spite of their different roles, there are several similarities between the periplasmic nitrate reductase systems of different species. Many have four proteins in common: NapA, NapB, NapC (or a homologue) and NapD. NapA is a periplasmic protein that binds a molybdenum *bis*-molybdopterin (Mo-*bis*-MGD) cofactor and a 4Fe4S cluster. The cofactors are incorporated into the enzyme in the cytoplasm and it is then exported as a folded protein via the twin-arginine translocase (TAT) system. NapB is a di-haem periplasmic protein that is exported by the Sec pathway. NapA and NapB then interact in the periplasm, with the site of nitrate reduction on the NapA Mo-*bis*-MGD cofactor and NapB functioning as an electron donor to NapA. NapC is a tetra-haem cytochrome that is anchored into the cytoplasmic membrane and is believed to be the quinol dehydrogenase of the system (Berks *et al.*,

Abbreviations: MK, menaquinone; TAT, twin-arginine translocase; UQ, ubiquinone; X-P, 5-bromo-4-chloro-3-indolyl phosphate.

1995a; Simon *et al.*, 2003; Brondijk *et al.*, 2004). NapD is located in the cytoplasm (Berks *et al.*, 1995a) and may play a role in NapA maturation prior to export (Potter & Cole, 1999).

In addition to these four proteins, many *nap* operons also encode additional proteins, mostly of unknown function. For example, in *E. coli* Nap is encoded by the *napFDAGHBC* operon. Based on their primary structure, NapF, NapG and NapH are all believed to be non-haem iron-sulphur proteins. We recently reported that NapH is an integral membrane protein with four membrane-spanning helices (Brondijk *et al.*, 2004). The N- and C-termini, as well as the two iron-sulphur centres, are located in the cytoplasm. NapG is loosely attached on the periplasmic side of the membrane, and is transported across the membrane by the TAT pathway (Brondijk *et al.*, 2004; Santini *et al.*, 1998; Weiner *et al.*, 1998; Berks *et al.*, 2000). NapG and NapH are both required for optimal electron transfer from ubiquinol to the periplasmic Nap complex (Brondijk *et al.*, 2004). The location of NapF has been the subject of debate, with early contradictory schemes placing it in either the periplasm (Berks *et al.*, 1995a) or the cytoplasm (Reyes *et al.*, 1998). This has been because its N-terminal sequence includes two arginine residues in a motif with some similarity to a TAT export signal (Weiner *et al.*, 1998; Berks *et al.*, 2000) that suggests a periplasmic location (Reyes *et al.*, 1998). However, NapF lacks other N-terminal features typical of proteins secreted via the TAT pathway, which points to a cytoplasmic location (Berks *et al.*, 1995a). Only two *napF* mutants have so far been characterized, one in *Rhodobacter sphaeroides*, in which NapF is essential for nitrate reduction (Reyes *et al.*, 1998), the other in *E. coli*, in which loss of NapF function results in subtle nitrate-dependent growth defects, especially during adaptation from aerobic to anaerobic growth (Brondijk *et al.*, 2002). Experimental evidence has recently emerged for the *R. sphaeroides* NapF that supports a cytoplasmic location (Olmo-Mira *et al.*, 2004), but further data from other organisms is required to establish whether this is also true for other bacteria. This is provided in the current work, which presents evidence that NapF is indeed located in the cytoplasm and provides the first *in vivo* evidence that it can interact with NapA in this subcellular compartment. Since mature NapA is located in the periplasm, this points to a role for NapF in interaction with NapA during the maturation of the enzyme in the cytoplasm prior to export of the mature form into the periplasm.

METHODS

Strains, plasmids and growth conditions. Strains of *Escherichia coli* K-12 and plasmids used or constructed during this study are listed in Table 1. Strains JCB4141 and 4142 lack both of the cytoplasmic nitrate reductases, A and Z, and, due to the *narL* mutation, overexpress the periplasmic nitrate reductase during anaerobic growth in the presence of nitrate. Anaerobic growth of these strains in minimal medium in the presence of a non-fermentable carbon source such as glycerol requires a reducible electron acceptor, so

growth with nitrate is Nap-dependent (Brondijk *et al.*, 2002). Bacteria were grown aerobically in LB (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 5 g NaCl l⁻¹) or anaerobically in MS medium [4.5 g KH₂PO₄ l⁻¹, 10.5 g K₂HPO₄ l⁻¹, 1 g NH₄SO₄ l⁻¹, 0.05 g MgCl₂ l⁻¹, 2.5 g nutrient broth (NB) base l⁻¹, 10 µM sodium molybdate, 10 µM sodium selenate and 1 ml l⁻¹ of *E. coli* sulphur-free salts (Pope & Cole, 1984) supplemented with 20 mM NaNO₃ and 0.4% (v/v) glycerol or 0.4% (w/v) D-glucose]. When appropriate 100 µg ampicillin ml⁻¹ and/or 50 µg kanamycin ml⁻¹ were added. Unless stated otherwise, cultures were grown at 37 °C.

Construction of the pAN100 series of plasmids. Primers used for cloning steps are listed in Table 2. The cloning vector for the pAN100 series of plasmids was pBR322 (Bolivar *et al.*, 1977). The DNA sequence of the *napF* promoter and coding region was amplified by PCR using plasmid pJG460 (Grove *et al.*, 1996) as a template. Two primers, pNapFEcoRI and NapFBamH2, containing *EcoRI* and *BamHI* at the end of each primer, respectively, were used to create the 900 bp DNA fragment. The PCR fragment was digested with *EcoRI* and *BamHI* and then ligated into the *EcoRI*-*BamHI* site of pBR322. The resulting plasmid, pAN101, contains the complete DNA sequence of *napF*, expressed under the control of the *nap* promoter.

Two highly conserved sequence motifs in the NapF protein family are a double-arginine motif close to the N-terminus, and the unusual sequence I-R-P-P-W 12–20 residues downstream. Both motifs are also present in NapG, which was previously shown to be exported into the periplasm (Brondijk *et al.*, 2004). As highly conserved sequences are often essential for NapF function, the double-arginine motif in the putative signal sequence of NapF was changed to a double alanine using the Quikchange Site-Directed Mutagenesis kit (Stratagene). First a megaprimer was generated by PCR using primer NapF BamH2 and the mutagenic primer NapF RR to AA with pJG600 as template. The purified 500 bp product was then used in a PCR reaction with primer pNapF *EcoRI* and pJG600 as template. The 900 bp product was cloned into pBR322 to yield plasmid pAN103. Similarly, the proline motif in NapF was modified using the primers listed in Table 2 to generate the derivatives pAN111, encoding the substitution P24A, pAN112 (P25A), pAN113 (W26Y) and pAN114, encoding all three substitutions (P24A P25A W26Y).

Construction of the pLP800 series of plasmids. Plasmid pLP802 was constructed by amplifying a DNA fragment containing *napF* using primers NapFNdeI, which created an *NdeI* restriction site upstream from the start codon of *napF*, and NapFBamH2, which created a *BamHI* restriction site at the end of *napF*. The PCR fragment was then restricted with *NdeI* and *BamHI* and cloned into pET11c vector. Plasmid pLP802 expresses NapF under the control of an inducible T7 promoter. To create plasmid pLP804, which expresses *napF* with a *myc* epitope, the *napF* gene was amplified using primers NapFNdeI and NapFmycBamHI. The latter primer creates a Myc tag at the C-terminus of NapF and *BamHI* restriction site immediately after the stop codon. The *NdeI*/*BamHI*-digested fragment was then cloned into pET11c. Plasmid pLP805 was constructed to contain the *napF* gene with a mutation at the putative twin-arginine motif. The mutagenic primer NapFRRtoAA was designed to contain the sequences that encoded two alanines instead of two arginines at the conserved position and an *NdeI* site was created. The mutated *napF* gene was amplified using primers NapFRRtoAA and NapFmycBamHI and subsequently cloned into pET11c (as described above). Plasmid pLP805 expresses *napF* with mutations at the conserved twin-arginine motif and a *myc* tag from the T7 promoter.

Construction of plasmids and detection of PhoA and LacZ fusion proteins. The *napF* gene, including its promoter but without the translational termination codon, was amplified by PCR

Table 1. *E. coli* strains and plasmids

Strain/plasmid	Genotype/description	Reference/source
Strains		
BL21(DE3)pLysS	F ⁻ <i>omp hsdS_B (r_B⁻ m_B⁻) gal dcm</i> (DE3)pLysS (Cm ^R)	Novagen
BTH101	F ⁻ <i>cya-99 araD139 galE15 galK16 rpsL1</i> (Str ^R) <i>hsdR2 mcrA1 mcrB1</i>	D. Ladant, Institut Pasteur
JCB4141	RK4353 $\Delta narZ :: \Omega \Delta narG-I \Delta narL :: Tn10 \Delta menBC$	Brondijk <i>et al.</i> (2002)
JCB4142	JCB4141 $\Delta napF$	Brondijk <i>et al.</i> (2002)
TOP10	F ⁻ <i>mcrA</i> $\Delta(mrr-hsd RMS-mcrBC)$ $\phi 80 lacZ \Delta M15 \Delta lacX74 deoR recA1 araD139 \Delta(araA-eu)7697 galU galK rpsL endA1 nupG$	Invitrogen
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lac^aZ</i> $\Delta M15$ Tn10 (Tet ^R)]	Stratagene
Plasmids		
pBR322	4.36 kb Amp ^R Tet ^R medium-copy-number cloning vector with ColE1 origin of replication	
pAN101	900 bp PCR fragment encoding <i>napF</i> from pJG460 ligated as an <i>EcoRI</i> – <i>Bam</i> HI fragment into pBR322	This study
pAN103	Derivative of pAN101 with the twin Arg codons changed by QuikChange site-directed mutagenesis to Ala codons	This study
pAN105A	900 bp <i>napF</i> PCR fragment cloned as a <i>Pst</i> I– <i>Bam</i> HI fragment into the <i>phoA</i> vector, pSK4158	This study
pAN105Z	900 bp <i>napF</i> PCR fragment cloned as an <i>EcoRI</i> – <i>Bam</i> HI fragment into the <i>lacZ</i> vector, pNM480	This study
pAN111	Derivative of pAN101 with the Pro-24 codon changed by QuikChange site-directed mutagenesis by an Ala codon	This study
pAN112	Derivative of pAN101 with the Pro-25 codon changed by QuikChange site-directed mutagenesis by an Ala codon	This study
pAN113	Derivative of pAN101 with the Trp-26 codon changed by QuikChange site-directed mutagenesis to a Tyr codon	This study
pAN114	Derivative of pAN101 modified by QuikChange site-directed mutagenesis to encode the P24A P25A W26Y substitutions	This study
pET11c	5.7 kb Amp ^R pBR322-based plasmid containing T7 <i>lac</i> promoter	Novagen
pJG460	10 kb <i>Bam</i> HI fragment from pJG600 containing upstream region of the <i>nap-ccm</i> operon in pAA182 <i>Bam</i> HI	Grove <i>et al.</i> (1996)
pKT25	3.44 kb Km ^R pSU40 derivative plasmid expressing the first 224 aa (T25) of CyaA with the multiple cloning site at the 3' end of T25	D. Ladant, Institut Pasteur
pKT25-A	2.5 kb <i>napA</i> gene ligated into pKT25; promoter <i>plac</i> expresses T25-NapA hybrid protein	This study
pKT25-C	600 bp <i>napC</i> gene generated by PCR with <i>Xba</i> I– <i>Kpn</i> I 5' overhangs, ligated into pKT25 at <i>Xba</i> I and <i>Kpn</i> I sites; promoter <i>plac</i> expresses T25-NapC hybrid protein	Brondijk <i>et al.</i> (2004)
pKT25-D	270 bp <i>napD</i> ligated into pKT25; promoter <i>plac</i> expresses T25-NapH hybrid protein	This study
pKT25-F	500 bp <i>napF</i> gene generated by PCR with <i>Xba</i> I– <i>Kpn</i> I 5' overhangs, ligated in pKT25 <i>Xba</i> I– <i>Kpn</i> I; promoter <i>plac</i> expresses T25-NapF hybrid protein	Brondijk <i>et al.</i> (2004)
pKT25-G	700 bp <i>napG</i> ligated into pKT25; promoter <i>plac</i> expresses T25-NapH hybrid protein	This study
pKT25-H	860 bp <i>napH</i> gene generated by PCR with <i>Xba</i> I– <i>Kpn</i> I 5' overhangs, ligated in pKT25 <i>Xba</i> I– <i>Kpn</i> I; promoter <i>plac</i> expresses T25-NapH hybrid protein	Brondijk <i>et al.</i> (2004)
pMN480	8.6 kb Amp ^R <i>lacZ</i> fusion vector	Minton (1984)
pSK4158	4.8 kb Cm ^R <i>phoA</i> fusion vector	Paulsen <i>et al.</i> (1995)
pLP802	500 bp PCR fragment containing the <i>napF</i> gene with <i>Nde</i> I and <i>Bam</i> HI 5' overhangs ligated into pET11c at <i>Nde</i> I and <i>Bam</i> HI sites	This study
pLP804	530 bp PCR fragment containing the <i>napF</i> gene with a <i>myc</i> tag at the 3' end ligated into the <i>Nde</i> I and <i>Bam</i> HI sites of pET11c	This study
pLP805	As pLP804, but with the double Arg codons replaced by Ala codons	This study
pUT18	3.02 kb Amp ^R pUC19 derivative plasmid expressing the T18 fragment (amino acids 225–399) of CyaA with the multiple cloning site at the 5' end of T18	D. Ladant, Institute Pasteur
pUT18-A	2.5 kb <i>napA</i> gene ligated into pUT18; promoter <i>plac</i> expresses NapA-T18 hybrid protein	This study
pUT18-C	600 bp <i>napC</i> gene generated by PCR with <i>Xba</i> I– <i>Kpn</i> I 5' overhangs, ligated into pUT18 at <i>Xba</i> I and <i>Kpn</i> I sites; promoter <i>plac</i> expresses NapC-T18 hybrid protein	Brondijk <i>et al.</i> (2004)

Table 1. cont.

Strain/plasmid	Genotype/description	Reference/source
pUT18-D	270 bp <i>napD</i> gene ligated into pUT18; promoter <i>plac</i> expresses NapD-T18 hybrid protein	This study
pUT18-F	500 bp <i>napF</i> gene generated by PCR with <i>XbaI</i> - <i>KpnI</i> 5' overhangs, ligated in pUT18 <i>XbaI</i> - <i>KpnI</i> ; promoter <i>plac</i> expresses NapF-T18 hybrid protein	Brondijk <i>et al.</i> (2004)
pUT18-G	700 bp <i>napG</i> gene ligated into pUT18; promoter <i>plac</i> expresses NapG-T18 hybrid protein	This study
pUT18-H	860 bp <i>napH</i> gene generated by PCR with <i>XbaI</i> - <i>KpnI</i> 5' overhangs, ligated in pUT18 <i>XbaI</i> - <i>KpnI</i> ; promoter <i>plac</i> expresses NapH-T18 hybrid protein	Brondijk <i>et al.</i> (2004)
pUT18C	3.02 kb Amp ^R pUC19 derivative plasmid expressing the T18 fragment (amino acids 225–399) of CyaA with the multiple cloning site at the 3' end of T18	D. Ladant, Institut Pasteur
pUT18C-C	600 bp <i>napG</i> gene generated by PCR with <i>XbaI</i> - <i>KpnI</i> 5' overhangs, ligated into pUT18C at <i>XbaI</i> and <i>KpnI</i> sites; promoter <i>plac</i> expresses T18-NapC hybrid protein	Brondijk <i>et al.</i> (2004)
pUT18C-A	2.5 kb <i>napA</i> gene generated by PCR ligated into pUT18C; promoter <i>plac</i> expresses T18-NapA hybrid protein	This study
pUT18C-D	270 bp <i>napD</i> gene generated by PCR ligated into pUT18C; promoter <i>plac</i> expresses T18-NapD hybrid protein	This study
pUT18C-F	500 bp <i>napF</i> gene generated by PCR with <i>XbaI</i> - <i>KpnI</i> 5' overhangs, ligated in pUT18C <i>XbaI</i> - <i>KpnI</i> ; promoter <i>plac</i> expresses T18-NapF hybrid protein	Brondijk <i>et al.</i> (2004)
pUT18C-G	700 bp <i>napG</i> gene generated by PCR ligated into pUT18C at <i>XbaI</i> and <i>KpnI</i> sites; promoter <i>plac</i> expresses T18-NapG hybrid protein	This study
pUT18C-H	860 bp <i>napH</i> gene generated by PCR with <i>XbaI</i> - <i>KpnI</i> 5' overhangs, ligated in pUT18C <i>XbaI</i> - <i>KpnI</i> ; promoter <i>plac</i> expresses T18-NapH hybrid protein	Brondijk <i>et al.</i> (2004)
pEL6HisNapF	<i>napF</i> excised from pLP802 and cloned into the His ₆ -tag vector pET16B	This study

with primers that introduced a *PstI* or *EcoRI* site upstream of the *nap* promoter and a *BamHI* site at the fusion point (Table 2). The *PstI*-*BamHI* fragments were cloned into pSK4158 (Manoil, 1991) to create in-frame *phoA* fusions and the *EcoRI*-*BamHI* fragments into pNM480 (Minton, 1984) to create in-frame *lacZ* fusions. Transformants were plated onto selective LB agar with 40 µg 5-bromo-4-chloro-3-indolyl phosphate (X-P) ml⁻¹ or 40 µg X-Gal ml⁻¹. *PhoA* and β -galactosidase activities were measured as described previously (Karimova *et al.*, 1998; Ladant & Ullmann, 1999).

Preparation of periplasmic, cytoplasmic and membrane extracts from bacterial cultures. Cultures were grown aerobically in 1 l of LB and induced with 0.5 mM IPTG at OD₆₅₀ ≈ 0.5. After 4 h of induction, bacteria were harvested, washed with 50 mM Tris/HCl buffer pH 8 and resuspended in 15 ml of a freshly prepared solution of SET buffer (0.5 M sucrose, 5 mM EDTA and 50 mM Tris/HCl, pH 8.0) containing 600 µg lysozyme ml⁻¹ (Brondijk *et al.*, 2004). The resuspended pellet was incubated at 30 °C for 1 h to allow the release of periplasmic proteins. The cell suspension was then centrifuged at 12 000 g for 30 min to pellet the spheroplasts. The periplasmic fraction was dialysed against 1 mM Tris/HCl, pH 8.0, freeze-dried and resuspended in sterile distilled water. The spheroplasts were resuspended in 5 ml 50 mM Tris/HCl pH 8.0 and passed through a French pressure cell at 10 000 p.s.i. The slurry, consisting of unbroken bacteria, cell membranes and cytoplasm, was centrifuged at 5000 g for 10 min to sediment unbroken bacteria and inclusion bodies, and then at 170 000 g for 1 h to sediment the membrane fraction. Soluble cytoplasmic proteins in the supernatant were removed. The resulting membrane pellet was homogenized with 5 ml 50 mM Tris/HCl pH 8.0 and further purified on a 36–54% (w/v) sucrose gradient to ensure that it was free from unbroken bacteria, outer membranes and inclusion bodies.

Purification of His₆-NapF and demonstration of the pull-down of NapA from soluble fractions of *E. coli*. One-litre

batches of *E. coli* BL21(DE3)pLysS containing the His₆-NapF expression vector pELHisNapF were grown up aerobically on LB medium at 37 °C. When the OD₆₀₀ reached 0.6 the cells were induced with 0.5 mM IPTG and the cultures incubated at 25 °C for a further 4 h. The cells were then harvested by centrifugation and a soluble extract was prepared by French pressure cell treatment of the cell pellet followed by resuspension in 20 mM Tris/HCl buffer pH 7.5. Purification of the His₆-NapF protein then involved sequential application of Ni-affinity, DEAE anion-exchange and S200 gel-exclusion chromatographies, followed by a second Ni-affinity step. The DEAE column was developed with a 0–500 mM NaCl gradient and NapF eluted at around 300 mM NaCl. The Ni-affinity columns were developed with 10–500 mM imidazole gradients and His₆-NapF eluted at around 480 mM imidazole. In all cases NapF-containing fractions were detected by SDS-PAGE analysis of column fractions.

To demonstrate pull-down of NapA by NapF, soluble fractions were prepared from *E. coli* grown anaerobically on a glycerol/nitrate minimal medium and applied to Ni-affinity columns that were either charged with His₆-NapF or left uncharged. Columns were washed with 20 mM Tris/HCl and 10 mM imidazole buffer pH 7.5, and then a 10–500 mM imidazole gradient in 20 mM Tris/HCl buffer pH 7.5 was applied in a total volume of 60 ml. Column eluate fractions were subjected to SDS-PAGE and stained with Coomassie brilliant blue or Western blotted and then probed with anti-NapA antibodies.

SDS-PAGE, Western blotting and N-terminal sequencing.

For SDS-PAGE, a 15% (w/v) resolving gel and a 6% (w/v) stacking polyacrylamide gel in an 18 cm square-slab apparatus were used exactly as described previously (Brondijk *et al.*, 2004). For whole-cell samples, the bacterial pellet was resuspended in sample buffer containing 12% (v/v) 2-mercaptoethanol and heated at 95 °C for 10 min prior to loading. Protein bands were stained with Coomassie brilliant blue or transferred to a PVDF membrane for Western blot

Table 2. Oligonucleotide primers

Key: +, coding strand; – complementary strand. Incorporated restriction sites are underlined; bases changed by site-directed mutagenesis are shown in bold; the *myc* tag sequence is shown in *italic*.

Primer name	Strand	Restriction site	Sequence (5'–3')
NapFNdeI	+	<i>NdeI</i>	TATTC <u>CCATATGA</u> AGATTGATGCATCCCG
NapFBamH2	–	<i>BamHI</i>	ATAATAGGATCCTCATTAGTGTGCATGGAGATACTCCG
NapF myc BamHI	–	<i>BamHI</i>	ATAATAGGATCCTTACAGATCCTCTTCTGAGATGAGTTTT- TGTTGCGTGTGCATGGAGATACTCCG
pNapFEcoRI	+	<i>EcoRI</i>	GAACGAATTCCCGATGATGTCGCTG
NapF RRtoAA	+	<i>NdeI</i>	TATTATCATATGAAGATTGATGCATCCGCGGCGGGCATA- CTCACTGGTCGC
NapF(P1toA)UP	+	–	GGTATCCGTGCGCCCTGGTCCGG
NapF(P1toA)DOWN	–	–	GGACCAGGGCGCACGGATACCG
NapF(P2toA)UP	+	–	ATCCGTCCGGCGTGGTCCGGTG
NapF(P2toA)DOWN	–	–	ACCGGACCACGCCGACGGATACCG
NapF(WtoY)UP	+	–	ATCCGTCCGCCCTATTCCGGTGATG
NapF(WtoY)DOWN	–	–	ATCACCGAATAGGGCGGACGG
NapF(PPWtoAAY)UP	+	–	AGTAACGGTATCCGTGCGGCGTATTCGGTGATG
NapF(PPWtoAAY)DOWN	–	–	ATCACCGAATACGCCGCACGGATACCGTTACTGG
Two-hybrid interaction primers			
NapAXbaI	+	<i>XbaI</i>	TATATTCTAGAGATGAACTCAGTCGTCGTAGC
NapAKpnI	–	<i>KpnI</i>	ATAAGGTACCCGCACCTTCTCCAGTTTGACCGC
NapAKpnIstop	–	<i>KpnI</i>	ATAAGGTACCCGTTACACCTTCTCCAGTTTGAC
NapDXbaI	+	<i>XbaI</i>	TATATTCTAGAGATGCACACTAACTGGCAAG
NapDKpnI	–	<i>KpnI</i>	ATAAGGTACCCGTGGTGTTCCTCACCTTGCTC
NapDKpnIstop	–	<i>KpnI</i>	ATAAGGTACCCGTGCATGGTGTTCCTCACCTTGC
NapGXbaI	+	<i>XbaI</i>	TATTATACTCTAGAGATGTCCCGGTGAGCGAAACC
NapGKpnI	–	<i>KpnI</i>	ATAATAAGGTACCCGCGATTTGCCATTGTTCCC
NapGKpnIstop	–	<i>KpnI</i>	ATAATAAGGTACCCGTCATTACGATTGTCATTGTTCCC
phoA-lacZ fusion primers			
pNapFPstI	+	<i>PstI</i>	TATACTGCAGCCGATGATGTCGCTGAAGG
NapFBamHI (w/o stop)	–	<i>BamHI</i>	AATAGGATCCTGTGCATGGAGATACTCCGC

analysis. Anti-Myc antibody was purchased from Invitrogen and anti-mouse IgG was obtained from TROPIX. The antibodies were detected using a chemiluminescent detection system (TROPIX). For the N-terminal sequencing, proteins were transferred on to PVDF membrane and stained with Coomassie brilliant blue. Bands of interest were excised and used directly for automated degradation sequencing by Alta Bioscience (University of Birmingham).

Bacterial two-hybrid system. The coding sequences of each of the *nap* genes (*napF*, *napD*, *napA*, *napG*, *napH* and *napC*), with or without the stop codon, as appropriate, were cloned in-frame into pUT18, pUT18C and pKT25. These plasmids encode fusion proteins with the T18 and T25 fragments of *Bordetella pertussis* adenylate cyclase (Karimova *et al.*, 1998, 2000; Ladant & Ullmann, 1999). Two complementary plasmids were transformed into the adenylate-cyclase-deficient (*cya*) *E. coli* strain BTH101. Transformants were plated on MacConkey agar supplemented with 1% maltose and incubated at 30 °C. Development of red colonies, indicative of a positive interaction, was followed for 72 h. The strength of the interactions was determined by measuring the β -galactosidase activity of the transformants after anaerobic growth to an OD₆₅₀ of 0.5 in LB supplemented with 20 mM NaNO₃ and 0.4% glycerol.

Rates of nitrate reduction by physiological substrates and reduced methylviologen. A nitrate electrode was used to measure

rates of nitrate reduction by washed bacterial suspensions incubated with 1.2 mM nitrate and the physiological substrates glucose, glycerol or formate (Pope & Cole, 1984). Nitrate reductase activities of permeabilized bacteria incubated with 1 mM nitrate and reduced methylviologen were determined as described previously (Potter & Cole, 1999).

Chemical assays. Aerobic and anaerobic cultures were grown and assayed for β -galactosidase activity as described previously (Jayaraman *et al.*, 1987). Nitrite concentrations were measured according to Pope & Cole (1984). Protein concentrations were determined by the Lowry method.

RESULTS

The subcellular localization of NapF

To initiate investigation of the subcellular location of NapF, the complete coding sequence for *napF* was fused in-frame to *phoA* in plasmid pSK4158, and to *lacZY* in plasmid pNM480, to generate plasmids pAN105A and pAN105Z, respectively (Table 1). The fusion plasmids were transformed into *E. coli* TOP10, and transformants were

plated onto nutrient agar supplemented with chromogenic substrates, either X-P or X-Gal. Both of the untransformed control strains, as well as bacteria transformed with the *phoA* fusion, pAN105A, formed white colonies on X-P plates (Table 3). In contrast, although the untransformed TOP10 formed white colonies on X-Gal, bacteria transformed with pAN105Z formed blue colonies. After anaerobic growth in LB supplemented with glycerol, nitrate and appropriate antibiotics, no alkaline phosphatase activity was detected in the pAN105A transformant, but the β -galactosidase activity of the pAN105Z transformant was 820 nmol ONPG hydrolysed min⁻¹ (mg dry mass)⁻¹ compared with 85 units in the untransformed control (Table 3). Since β -galactosidase is regarded as a marker for cytoplasmic enzymes and alkaline phosphatase is regarded as a marker of periplasmic enzymes these data support the view that NapF is located in the cytoplasm.

The use of gene fusions alone can give ambiguous results on subcellular location of TAT-dependent enzymes since *PhoA* is a poor substrate for the TAT (Stanley *et al.*, 2002). Consequently the subcellular location of NapF was also assessed using antibody-based approaches. To facilitate this, *E. coli* BL21(DE3)pLysS derivatives harbouring the plasmids pLP802 and pAN804, encoding NapF and a NapF-Myc fusion protein, respectively, were constructed (Table 1). To avoid disruption of any signal peptide that might be required for export to the periplasm, NapF was tagged with the Myc epitope at its C-terminus. This tagging approach was used to demonstrate the periplasmic location of the TAT-dependent NapG protein (Brondijk *et al.*, 2004). Cultures were initially grown aerobically at 37 °C in LB medium and induced at an OD₆₅₀ of 0.5 by the addition of 0.5 mM IPTG. Proteins in samples removed at intervals of 1 h were separated by SDS-PAGE. An intense 18 kDa band corresponding to NapF was detected 2 or 3 h after induction, but not before induction, in the bacteria transformed with pLP802 (Fig. 1a). This was replaced by a 20 kDa band in bacteria transformed with pLP804 (Fig. 1a). This 2 kDa shift in molecular mass was expected, as a result of the presence of the Myc epitope. N-terminal sequencing of the excised bands established the sequence

Met-Lys-Ile-Asp-Ala, which corresponds to the primary translation product without cleavage of an N-terminal leader sequence. This confirmed that these bands were indeed NapF and NapF-Myc and that the protein produced from pLP804 was suitable for use in further analyses in subcellular fractionation experiments.

For these fractionation experiments, cultures were grown at 25 °C in anaerobic, minimal salts medium supplemented with glucose and fumarate, a condition that minimized formation of inclusion bodies. After overnight growth in 2 l medium, NapF-Myc synthesis was induced by the addition of 0.5 mM IPTG; the bacteria were harvested after a further 4 h and fractionated into periplasm, cytoplasm and cytoplasmic membrane fractions. After SDS-PAGE and Western blotting of equal amounts of protein in each fraction, NapF-Myc was clearly detected in the cytoplasm and membrane fractions of induced bacteria (Fig. 1b). No NapF-Myc was detected in the periplasmic fraction (Fig. 1b). NapF-Myc in the cytoplasm and membrane fractions migrated with the same apparent mass of 20 kDa as the uncleaved, full-length NapF-Myc. NapF in the membrane fraction could be readily solubilized by treatment of the membranes with 0.1 % Triton X-100, 6 M urea or 0.2 % sodium deoxycholate and retained the same apparent mass of 20 kDa that was characteristic of the water-soluble cytoplasmic material. These subcellular localization experiments are fully consistent with the results of the gene fusion experiments and together the two approaches provide unequivocal evidence that NapF is a cytoplasmic protein loosely attached to the inner side of the membrane.

It is notable that NapF shares a high degree of sequence identity with NapG, which we have previously shown has a functional TAT signal sequence that targets it to the periplasm (Brondijk *et al.*, 2004). Primary structure analysis of *E. coli* NapF reveals the sequence SRRGILT close to the N-terminus, which is similar to the sequence SRRxFLK that is typical of leader peptides of proteins secreted into the periplasm by the TAT pathway. Multiple sequence analysis of NapF proteins from a range of proteobacteria reveal that this twin-arginine sequence is highly conserved, suggesting

Table 3. Enzyme activities of NapF-PhoA/LacZ fusions

Enzyme activities are nmol X-Gal or X-P hydrolysed min⁻¹ (mg dry weight)⁻¹. The data are means $\pm$ SD (*n*=3).

Plasmid	Colour on X-Gal or X-P indicator plates	β -Galactosidase or alkaline phosphatase activity
LacZ fusion		
pNM480 (No fusion)	White	85 ($\pm$ 2)
pAN105Z (NapF-LacZ)	Blue	820 ($\pm$ 170)
PhoA fusion		
pSK4158 (No fusion)	White	4 ($\pm$ 1)
pAN105A (NapF-PhoA)	White	5 ($\pm$ 1)

Fig. 1. Overexpression of NapF and NapF-Myc in *E. coli* BL21(DE3)pLysS and localization of the NapF and NapF R7A:R8A proteins. (a) SDS-PAGE analysis and staining by Coomassie brilliant blue of whole-cell samples. Cultures of *E. coli* BL21(DE3)pLysS/pLP802 (NapF) and *E. coli* BL21(DE3)pLysS/pLP804 (NapF-Myc) were induced with IPTG and grown aerobically for 3 h at 37 °C. The whole-cell samples were lysed and separated on a 15% SDS-polyacrylamide gel. (b, c) Cultures of *E. coli* BL21(DE3)pLysS/pLP804 expressing wild-type NapF (b) and BL21(DE3)pLysS/pLP805 expressing NapF R7A:R8A (c) were grown anaerobically at 25 °C and induced with IPTG. After 4 h incubation, cells were harvested and fractionated into periplasm, cytoplasm and membrane. The following samples were separated by 15% SDS-PAGE and analysed by Western blotting using anti-Myc antibodies and enhanced chemiluminescence: CA, whole cells after induction; P, periplasm (50 µg protein); C, cytoplasm (50 µg protein); M, membrane fraction after French pressing and purification on a sucrose density gradient (50 µg protein).

that it has functional significance. However, a hydrophobic region typical of TAT-dependent leader peptides does not follow it. Since the experiments described above establish that NapF is a cytoplasmic protein, the 'RR' motif conserved in many NapF proteins might simply be a remnant of the TAT motif that is not essential for NapF function. This was investigated by changing the codons for the double-arginine motif to alanine codons by site-directed mutagenesis. To develop an *in vivo* assay for NapF function we took advantage of our earlier observation that NapF is essential for nitrate-dependent growth of an *E. coli* strain defective in naphthoquinone synthesis. This strain, JCB4141, is defective in nitrate reductase A, nitrate reductase Z and menaquinone (MK) synthesis. Strain JCB4141 adapted slowly when transferred from aerobically grown LB pre-cultures to

an anaerobic minimal medium with glycerol as the sole carbon source and 20 mM nitrate as the only terminal electron acceptor (Fig. 2a). This growth exhibited a clear biphasic character, with a slow first phase (0–10 h) and faster second phase (10–20 h). This slow phase was not apparent when the adapted cultures were further subcultured into the same anaerobic growth conditions. The growth was totally dependent upon the addition of nitrate. The *napF* derivative strain JCB4142 consistently exhibited a low rate of growth after transfer from the aerobic to anaerobic culture conditions but could not adapt to the new growth conditions and enter into the second fast growth phase (Fig. 2a), suggesting that NapF was needed to facilitate this adaptation. The second growth phase of JCB4142 was fully restored by transformation with plasmid pAN101, in which *napF* is cloned into pBR322 (Fig. 2b). This then established a functional growth-based assay for active NapF.

The modified *napF* gene encoding NapF in which the RR motif was substituted by AA was amplified by PCR and cloned into pBR322 to generate plasmid pAN103 (Table 1). This plasmid was as effective as pAN101 in complementing the growth defect of strain JCB4142, establishing that the arginine motif is not essential for NapF function (Fig. 2b). These data also further established that NapF is not targeted to the periplasm by the TAT secretion system, as it would not have been expected to complement growth had it been targeted to the wrong subcellular compartment.

Plasmid pAN101 was also used as the template to introduce point mutations into a conserved R-P-P-W motif that is located 14 amino acids downstream of the RR motif of NapF. The derivatives generated were pAN111, encoding the substitution P24A, pAN112 (P25A), pAN113 (W26Y) and pAN114 (P24A P25A W26Y) (Table 1). All of the JCB4142 transformants with these plasmids grew at the same rate as JCB4142 transformed with pAN101, and grew slightly more rapidly than the isogenic *napF*⁺ strain JCB4141 transformed with the vector plasmid, pBR322 (Fig. 2c). It was concluded that the R-P-P-W motif, like the highly conserved double-arginine motif, is not essential for NapF function.

***In vitro* and *in vivo* protein–protein interaction between NapF and NapA**

The cytoplasmic location of NapF is consistent with a role in maturation of NapA prior to export of the enzyme to the periplasm via the TAT pathway. To demonstrate that NapF could interact with NapA, a His₆-NapF protein was constructed for use in NapA pull-down assays. The *napF* gene was excised from pLP802, cloned into the His₆-tag vector pET16B (to generate pELHisNapF, Table 1) and expressed in *E. coli* BL21(DE3)pLysS. His₆-NapF was purified from soluble fractions by sequential Ni-affinity, DEAE anion-exchange and S200 gel-exclusion chromatographies, followed by a second Ni-affinity step. The His₆-NapF protein eluted at around 480–500 mM imidazole on

Fig. 2. Growth curve assays of NapF functionality. (a) Effect of deletion of the *napF* gene on Nap-dependent growth of strains producing only UQ. Strains were grown anaerobically in MS medium supplemented with 10% (v/v) NB and glycerol in the presence or absence of nitrate. The cell densities were measured every hour for 24 h. $\blacktriangle$, JCB4141 (Nap⁺; no nitrate); $\bullet$, JCB4141 (Nap⁺; nitrate added); $\triangle$, JCB4142 (Δ *napF*; nitrate added). The data are representative of three independent experiments. (b) Growth complementation of the *napF* deletion strain with NapF expressed from a plasmid. Growth of strains expressing Nap as the only nitrate reductase and defective in MK synthesis. $\bullet$, pBR322 in JCB4141 (Nap⁺); $\circ$, pBR322 in JCB4142 (Δ *napF*); $\triangle$, pAN101 in JCB4142 (Δ *napF*+NapF); $\blacktriangle$, pAN103 in JCB4142 [Δ *napF*+NapF (RR-AA)]. Strains were grown anaerobically in MS+10% NB supplemented with glycerol, nitrate and ampicillin. The data are representative of three independent experiments. (c) Effect of amino acid substitutions in the proline motif of NapF on growth. Growth of strains expressing Nap as the only nitrate reductase and defective in the MK synthesis. $\bullet$, pBR322 in JCB4141 (Nap⁺); $\circ$, pBR322 in JCB4142 (Δ *napF*); $\blacktriangle$, pAN101 in JCB4142 (Δ *napF*+plasmid-encoded NapF); $\square$, pAN111 (encoding NapF P24A) in JCB4142; $\blacksquare$, pAN112 (NapF P25A) in JCB4142; $\times$, pAN113 (NapF W26Y) in JCB4142; $\triangle$, pAN114 (NapF P24A:P25A:W26Y) in JCB4142. Transformants were grown anaerobically in minimal salts medium supplemented with 10% (v/v) NB, glycerol, nitrate and ampicillin. The data are representative of two independent growth experiments. Note the linear rather than exponential y-axis, which, because the exponential phase of growth was rather short, was used to emphasize the effects of nitrate and *napF* mutations on growth.

the Ni-affinity columns and was around 95% pure, as judged by Coomassie blue staining of SDS-PAGE gels, after the four steps. Tracking the NapF-containing fractions by UV-visible spectroscopy during purification revealed enrichment of a protein with the spectral properties of an iron-sulphur protein, as indicated by characteristic broad absorption features between 600 and 350 nm. This absorption spectrum was consistent with previous amino acid sequence analysis that has suggested that NapF is an iron-sulphur protein. However, the intensity of these bands varied from preparation to preparation and they were lost on storage of the protein at 4 °C or -20 °C. This prevented reproducible quantification and suggested that the iron-sulphur clusters bound by *E. coli* NapF were labile, a property that was also recently observed for the homologous NapF protein from the phototroph *R. sphaeroides* (Olmo-Mira *et al.*, 2004). This is also consistent with a preliminary report that an N-terminal fusion of the maltose-binding protein to NapF generated a protein that contained less than a stoichiometric quantity of iron and acid-labile sulphur in an approximately 1:1 ratio (Kim *et al.*, 2003).

To assess whether *E. coli* NapA could bind to immobilized His₆-NapF, soluble fractions were prepared from *E. coli*

grown anaerobically on a glycerol/nitrate minimal medium and applied to Ni-affinity columns that were either charged with His₆-NapF or left uncharged. Column eluate fractions were subjected to SDS-PAGE and Western blotting and then probed with anti-NapA antibodies. When soluble extracts were added to uncharged columns all of the NapA present in the soluble extract was eluted in the column wash; no binding of NapA to the column was detectable. However, when soluble extracts were applied to columns charged with His₆-NapF virtually no NapA eluted in the wash buffers. Almost all of the NapA in the soluble fractions bound to the column. This established that the immobilized NapF was retaining NapA on the Ni-affinity column. Following application of an imidazole elution gradient, the NapA remained tightly bound to the column until finally eluting at 480–500 mM imidazole, which coincided with the point of NapF elution.

The NapA in these subcellular fractions is present as the mature, folded and active periplasmic enzyme with all of the cofactors bound, and this is the form of NapA pulled down by NapF in these *in vitro* experiments. However, this periplasmic form of NapA is unlikely to be the form with which NapF interacts initially in the cytoplasm. Thus, whilst these experiments confirmed that NapF can interact with

folded NapA, other approaches were required to demonstrate that an interaction in the cytoplasm was possible. To this end a bacterial two-hybrid system was adopted. In this system, pairs of compatible plasmids are used to clone fragments of the gene for *B. pertussis* adenylate cyclase in-frame to the 5' or 3' end of the coding regions of the proteins to be investigated (Karimova *et al.*, 1998, 2000; Ladant & Ullmann, 1999). Interaction between two test proteins, or between a test protein and itself expressed from a second plasmid, results in adenylate cyclase activity that is revealed by activation of *lacZ* expression. Every combination of *napA*, *C*, *D*, *F*, *G* and *H* genes was tested in this system (Table 4). No interaction between NapF and either NapG or NapH was detected, consistent with our previous proposal that NapG and NapH form an ubiquinol dehydrogenase complex that transfers electrons from ubiquinol in the membrane to the periplasmic NapC. However, NapA apparently forms dimers or higher homopolymers, as revealed by high β -galactosidase activities when *napA* was cloned into two compatible pairs of plasmids. The self-association of NapC and its interaction with NapH were reported previously from a more qualitative plate-based assay (Brondijk *et al.*, 2004), as were the absence of interactions between the periplasmic NapG and other components of the Nap pathway (Brondijk *et al.*, 2004), and these are confirmed more quantitatively in the present set of analyses (Table 4). Although NapD and NapF are the only two Nap polypeptides that are located in the cytoplasm, no interactions between them were detected and neither NapD nor NapF appears to self-associate (Table 4). However, despite the fact that NapA is targeted by the TAT pathway to the periplasm, the two-hybrid assay suggests that both NapD and NapF interact strongly with NapA. As the two-hybrid system only detects interactions that occur in the

cytoplasm or on the cytoplasmic side of the membrane, it is inferred that NapA forms a transient complex with NapD and NapF in the cytoplasm before it is secreted as a mature enzyme by the TAT pathway.

DISCUSSION

The role of NapF in the periplasmic nitrate reductase system of bacteria has provided something of a conundrum. In *E. coli*, in the absence of the membrane-bound nitrate reductases NarA and NarX, NapA provides the only pathway for respiratory nitrate reduction. NapA is then essential for growth on non-fermentable carbon sources when nitrate is the only electron acceptor available (Brondijk *et al.*, 2002). Deletion of NapF lowers this growth rate, but does not abolish growth. However, NapF is absolutely essential for NapA-dependent growth of a strain that is defective in MK synthesis and therefore dependent on ubiquinone (UQ) for growth. The data presented in this paper show that *E. coli* NapF, like *R. sphaeroides* NapF, is located in the cytoplasm, where it can interact with NapA. Our study also provides the first direct evidence for an interaction in the cytoplasm not only between NapA and NapF, but also between NapA and NapD, which has previously been inferred (Berks *et al.*, 1995a; Reyes *et al.*, 1998; Potter & Cole, 1999) but never actually demonstrated. The detailed molecular and kinetic nature of the NapF–A and NapD–A interactions remain the subject of future work, but the interaction demonstrated here suggests a role for *E. coli* NapF, together with NapD, in the post-translational modification of NapA prior to its export to the periplasm via the TAT pathway. A possible clue to these functions is that both an iron–sulphur centre and Mo–bis-MGD cofactor must be incorporated into NapA before it is translocated via the TAT pathway and it is

Table 4. β -Galactosidase activities from Nap interactions using the bacterial two-hybrid assay

The β -galactosidase activities were assayed as described in Methods. The unit of activity is nmol min⁻¹ (mg dry weight)⁻¹. The data are means $\pm$ SD ($n=3$). Positive interactions are indicated in bold.

Protein	Plasmid	T25::NapC	T25::NapG	T25::NapH	T25::NapF	T25::NapD	T25::NapA
		pKT25-C	pKT25-G	pKT25-H	pKT25-F	pKT25-D	pKT25-A
NapC::T18	pUT18-C	100 (± 5)	132 (± 58)	97 (± 2)	106 (± 10)	86 (± 3)	98 (± 3)
T18::NapC	pUT18C-C	1820 (± 175)	110 (± 4)	180 (± 5)	112 (± 2)	87 (± 3)	80 (± 3)
NapG::T18	pUT18-G	97 (± 6)	88 (± 2)	100 (± 5)	41 (± 4)	88 (± 2)	91 (± 3)
T18::NapG	pUT18C-G	99 (± 15)	82 (± 2)	92 (± 4)	45 (± 9)	85 (± 4)	84 (± 3)
NapH::T18	pUT18-H	91 (± 5)	101 (± 1)	139 (± 62)	91 (± 1)	106 (± 6)	93 (± 4)
T18::NapH	pUT18C-H	232 (± 10)	98 (± 2)	89 (± 4)	84 (± 3)	109 (± 1)	81 (± 2)
NapF::T18	pUT18-F	116 (± 0.4)	51 (± 3)	85 (± 2)	38 (± 1)	139 (± 19)	2130 (± 652)
T18::NapF	pUT18C-F	112 (± 1)	51 (± 2)	84 (± 1)	42 (± 10)	142 (± 7)	2570 (± 1430)
NapD::T18	pUT18-D	89 (± 5)	78 (± 4)	106 (± 2)	146 (± 4)	144 (± 10)	4200 (± 690)
T18::NapD	pUT18C-D	88 (± 4)	83 (± 5)	97 (± 4)	161 (± 3)	139 (± 18)	3110 (± 380)
NapA::T18	pUT18-A	87 (± 7)	86 (± 1)	81 (± 9)	6320 (± 355)	7110 (± 570)	5300 (± 1100)
T18::NapA	pUT18C-A	83 (± 10)	85 (± 1)	87 (± 2)	3590 (± 140)	4760 (± 360)	1670 (± 100)

therefore possible that NapF facilitates the insertion of either one of these cofactors. In this respect the data presented here have some resonance with recent work with *R. sphaeroides* NapF that also suggested the presence of labile iron–sulphur clusters and showed that addition of NapF *in vitro* to inactive NapA, deficient in its iron–sulphur centre, reactivated the enzyme in the presence of cysteine and cysteine desulphhydrase (Olmo-Mira *et al.*, 2004).

If NapF is involved in the maturation of NapA in the cytoplasm, how can we rationalize the different phenotypes of *napF* mutants amongst bacterial species and the absence of *napF* from the *nap* operons of some species? This is the conundrum: *napF* is absent from the *nap* operon of *Paracoccus denitrificans* (Berks *et al.*, 1995b), but present in the *nap* operon of the closely related *R. sphaeroides*, where it is essential for the synthesis of active NapA (Reyes *et al.*, 1998). However, as discussed above, in *E. coli* deletion of *napF* only slows down adaptation from aerobic to anaerobic NapA-dependent growth of a UQ⁺/MK⁺ strain (Brondijk *et al.*, 2002), but is essential for NapA-dependent growth in a strain defective in MK synthesis (Brondijk *et al.*, 2004 and this study). In *E. coli*, during aerobic growth, UQ is the most abundant quinone pool (Unden, 1988; Soballe & Poole, 1999) and the *nap* operon is not expressed. Although electron transfer through the MK pool to the Nap complex in anaerobically adapted *E. coli* is much more rapid than through the UQ pool (Brondijk *et al.*, 2002, 2004), the opposite is likely to be true during the initial stages of adaptation, before adequate NapA and MK have accumulated (Brondijk *et al.*, 2002, 2004). Therefore any defect in NapA maturation is likely to be more apparent during this adaptation phase. These suggestions imply that, although NapF accelerates post-translational maturation of NapA, it is not strictly essential in *E. coli*. Possibly its function can be duplicated less efficiently by a component of another anaerobically expressed pathway, for example by a protein that, like NapF, is involved in cofactor insertion into the pre-apo-protein. This might also then be true in *P. denitrificans*, where there is no *napF* gene in the *nap* operon, but not in *R. sphaeroides*, where the *napF* of the *nap* operon is clearly essential (Reyes *et al.*, 1998).

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