Essential roles for the products of the *napABCD* genes, but not *napFGH*, in periplasmic nitrate reduction by *Escherichia coli* K-12

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The seven *nap* genes at minute 47 on the *Escherichia coli* K-12 chromosome encode a functional nitrate reductase located in the periplasm. The molybdoprotein, NapA, is known to be essential for nitrate reduction. We now demonstrate that the two *c*-type cytochromes, the periplasmic NapB and the membrane-associated NapC, as well as a fourth polypeptide, NapD, are also essential for nitrate reduction in the periplasm by physiological substrates such as glycerol, formate and glucose. None of the three iron–sulphur proteins, NapF, NapG or NapH, are essential, irrespective of whether the bacteria are grown anaerobically in the presence of nitrate or fumarate as a terminal electron acceptor, or by glucose fermentation. Mutation of *napD* resulted in the total loss of Methyl Viologen-dependent nitrate reductase activity of the molybdoprotein, NapA, consistent with an earlier suggestion by others that NapD might be required for post-translational modification of NapA.

Key words: *c*-type cytochromes, iron–sulphur proteins, *nap* genes, periplasmic nitrate reductase.

INTRODUCTION

In the absence of oxygen, *Escherichia coli* K-12 will preferentially use nitrate as a terminal electron acceptor for anaerobic growth. Nitrate is reduced to nitrite by three genetically and biochemically distinct enzymes. Nitrate reductase A is a heterotrimeric, membrane-bound enzyme encoded by the *narGHJI* operon [1,2]. It couples the oxidation of physiological substrates, especially formate [3,4], to the reduction of nitrate and generation of a proton gradient [5]. The active site of the enzyme is within the α subunit, NarG, which faces the cytoplasm and co-ordinates a molybdopterin guanine dinucleotide cofactor [6–8]. Electrons are sequentially transferred to the α subunit from the quinone pool via the γ subunit, a *b*-type cytochrome that is thought to contain the quinol oxidation site, and the β subunit which is a non-haem iron–sulphur protein [9]. Expression of nitrate reductase A is activated by the phosphorylated NarL protein (NarL-P) during anaerobic growth in the presence of nitrate [10,11].

Nitrate reductase Z, encoded by the *narZYWV* operon, probably arose by a duplication of the *narGHJI* locus, as its subunits are homologues of the nitrate reductase A subunits [12]. Biochemically it has similar properties to nitrate reductase A, but is expressed at very low level and is unaffected by anaerobiosis or the presence or absence of nitrate [12–14]. It has been proposed that nitrate reductase Z might facilitate adaptation from aerobic growth to anaerobic growth in the presence of nitrate [14].

The *E. coli* genome sequencing project revealed a number of genes in the minute-47 region of the chromosome that resembled those encoding the periplasmic nitrate reductase of *Thiosphaera pantotropha* [15,16]. This third nitrate reductase, Nap, is encoded by the *napFDAGHBC* operon and is biochemically very different from both nitrate reductase A and Z [17]. Immediately downstream of the *nap* genes are eight cytochrome *c* maturation genes, *ccmA–ccmH*, that are essential for the periplasmic assembly of *c*-type cytochromes during anaerobic growth [17,18]. Most of the transcription of the *ccm* genes originates at the *napF* promoter, which is induced by the fumarate and nitrate reductase regulatory protein (FNR) only during anaerobic growth [17]. Transcription is also induced by NarL and NarP in response to nitrite, but is repressed by NarL in the presence of nitrate [11,18]. There is a second, weaker *ccm* promoter located within the final *nap* gene, *napC* [18]. There is also a periplasmic nitrite reductase encoded by the *nrf* operon located at minute 92 on the chromosome [19,20]. The first two *nrf* genes, *nrfA* and *nrfB*, encode the 50 kDa periplasmic nitrite reductase, cytochrome c_{552}, and the pentahaem NrfB, cytochrome c_{550} [20]. Like the *nap* operon, transcription of the FNR-dependent *nrf* operon is induced during anaerobic growth by nitrite but is repressed by NarL in the presence of nitrate [11,19].

The *nap* operon in the aerobic denitrifier *T. pantotropha* [21,22] consists of five genes, *napEDABC*, and the enzyme has been thoroughly characterized [16] (reviewed by Berks et al. [23]). NapA is a 90 kDa molybdoprotein nitrate reductase, located in the periplasm [24]. The first crystal structure of a periplasmic nitrate reductase, that from *Desulfovibrio desulfuricans*, was recently reported [25]. The periplasmic dihaem *c*-type cytochrome, NapB, is thought to transfer electrons to NapA after receiving them from the proposed quinol oxidase, NapC, a tetrahaem, membrane-bound *c*-type cytochrome [26]. The NapA complex from another aerobic denitrifier, *Ralstonia* (formerly *Alcaligenes*) *eutrophus*, has also been characterized [27]. The functions of NapD and NapE are unknown, although it has been suggested that NapD might be involved in maturation of NapA [23]. Recently, the first experimental evidence for this proposal came from characterization of a strain of *Rhodobacter sphaeroides*, DSM158, that contained a non-polar mutation in the *napD* gene [28]. The absence of NapD resulted in the loss of about 95% of the nitrate reductase activity by this bacterium.

The variation between the Nap systems of different bacteria becomes evident when the operon structures are compared. All of the *nap* operons so far sequenced include four similar genes in the same order, *DABC*. This does not describe any known *nap* operon, however, because additional genes are always present.

Abbreviations used: FNR, fumarate and nitrate reductase regulatory protein; MS, minimal salts; LB, Luria–Bertani broth; NB, nutrient broth.

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For example, the *nap* operon of *R. sphaeroides* consists of seven genes in the order *napKEFDABC* [28,29]; *napK* has so far not been found in other bacteria and the function of its product is unknown. The *nap* operon of a rhizobium strain, previously designated *Pseudomonas* G179, is similar except that it lacks *napK* [30]. The *nap* operon of *E. coli* also includes *napF*, but lacks *napE* and *napK*. Instead, two other genes are present, *napG* and *napH*, located between *napA* and *napB*. This was the first published example of the *napDABC* unit being split. NapF, NapG and NapH are all predicted to be non-haem iron–sulphur proteins. The cellular location of NapF is unclear, as it does not have a recognizable signal sequence for export into the periplasm. NapG is predicted to be a soluble, periplasmic protein and NapH is likely to be a transmembrane protein with its iron–sulphur clusters facing the cytoplasm. The roles of these proteins in the Nap system of *E. coli*, and the physiological role or roles of Nap during anaerobic growth, are unknown. We have previously demonstrated that the seven *nap* genes of *E. coli* encode a functional, periplasmic nitrate reductase and that the molybdoprotein, NapA, is essential for activity [17,31]. We now report a systematic study of the effects of mutations in the six other *nap* genes on Nap activity after anaerobic growth under conditions of nitrate respiration, fumarate respiration and fermentation.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers used in the present work

Strains used were the derivatives of *E. coli* K-12 listed in Table 1. Plasmids and primers used in this work are listed in Tables 2 and 3 respectively. Plasmids encoding fragments of the *nap-ccm* operon are depicted in Figure 1. Stock antibiotics were added to liquid or molten agar medium to give the following final concentrations: 80 $\mu g \cdot ml^{-1}$ ampicillin; 30 $\mu g \cdot ml^{-1}$ chloramphenicol; 150 $\mu g \cdot ml^{-1}$ erythromycin; 50 $\mu g \cdot ml^{-1}$ spectinomycin; 50 $\mu g \cdot ml^{-1}$ kanamycin; and 15 $\mu g \cdot ml^{-1}$ tetracycline.

Amplification and use in strain construction of a DNA cassette encoding erythromycin resistance

The mutations in strains LP200 and JCB20480, both derivatives of LCB2048, were constructed by introduction of a cassette containing the *erm* gene from Tn1545 of *Streptococcus pneumoniae* [32] (GenBank® entry X52632). This gene encodes rRNA methylase that confers erythromycin resistance. Primers erm11 and erm22 were used to amplify this cassette with pMGC20 as template [33].

Table 1 *Escherichia coli* K-12 strains used in the present study

Strain	Description	Reference or source
JC7623	*recB21 recC22 sbcB15*	[42]
JCB20480	LCB2048 *napA*::ery	The present study
JM109	*endA1 recA gyrA96 thi hsdR17 relA1 supE44* λ- Δ(*lac-proAB*)	Promega
LCB320	*thi-1 thr-1 leu-6 lacY1 supE34 rpsL175*	[39]
LCB2048	LCB320 Δnar25(*narG-H*), Kan^R Δ*narU-Z*:: Ω	[39]
LP200	LCB2048 Δ*napG-ccmA*::ery	The present study
LP202	LCB2048 Δ*napD*	The present study
LP203	LCB2048 Δ*napF*	The present study
LP204	LP200 Δ*napF*	The present study

Table 2 Plasmids used in the present study

Plasmid	Description	Reference or source
pAA234	7 kb Amp^R pBR322-based plasmid containing the *galE* promoter followed by a *Bgl*II–*Sma*I–*Hin*dIII polylinker	[18]
pJG70	pBR322 derivative containing *napB-ccmH*	[17]
pJG260	pUC18 derivative expressing *napF-C* from *pnapF*	The present study
pJG267	As pJG260, but with an Ery^R cassette inserted in *napA*	The present study
pJG290	pUC18 derivative expressing *napF-ccmD* from *pnapF*	The present study
pJG292	As pJG290, but *napG* to *ccmA* replaced by Ery^R cassette	The present study
pJG350	*napC-ccmH* expressed from *pgalE* of pAA234	The present study
pJG351	*ccmA-H* expressed from *pgalE* of pAA234	[18]
pJG354	*napB-ccmH* expressed from *pgalE* of pAA234	The present study
pJG355	*napD* expressed from *pgalE* of pAA234	The present study
pJG600	Low-copy-number vector expressing *nap-ccm* operon from *pnapF*	[17]
pJG740	*napF-H* expressed from *pnapF* in pMAK705	The present study
pJG741	*napF-B* expressed from *pnapF* in pMAK705	The present study
pJG742	*napF-G* expressed from *pnapF* in pMAK705	The present study
pJG743	*napF-A* expressed from *pnapF* in pMAK705	The present study
pJG744	As pJG760, but with an in-frame deletion in *napF*	The present study
pJG745	As pJG760, but with an in-frame deletion in *napD*	The present study
pJG760	*napF-C* expressed from *pnapF* in pMAK705	The present study
pMAK705	Chlor^R low-copy-number vector; pBR322-compatible	[34]
pUC18	Amp^R high-copy-number vector	Pharmacia

Strain JCB20480 was constructed by cloning the *ery* fragment into the *napA* gene of plasmid pJG260 [17] to generate plasmid pJG267, which was linearized and transformed into the *recB recC sbcB* strain JC7623. The *napA*::*ery* mutation was transferred by bacteriophage P1 transduction into strain LCB2048 to give strain JCB20480.

Strain LP200 is a derivative of LCB2048, which contains a deletion of the four *nap* genes, *napGHBC*, as well as the *ccmA* gene. Plasmid pJG290 was constructed by subcloning the 8.5 kb *Pst*I–*Ssp*I fragment encoding the seven *nap* genes plus the first half of the *ccm* operon from pJG600 into pUC18 that had been digested with *Pst*I and *Sma*I. The 2.5 kb *Nru*I fragment from *napG* to *ccmA* of pJG290 was replaced by the 1 kb Ery^R cassette to generate plasmid pJG292. Plasmid pJG292 was linearized and transformed into strain JC7623. The Δ*napGHBC-ccmA* mutation was transferred by P1 transduction into strain LCB2048, resulting in strain LP200.

Table 3 Oligonucleotide primers used for the present study

+ refers to the coding strand; — to the complementary strand; *assignment with respect to transcription from *pnapF*. Incorporated restriction sites are underlined.

Primer	Location	Strand	Sequence (5′ to 3′)
NapDBamHI	*napF*	+	TTAGGATCCGCTGTCCGGTATCAGCC
napDHind3	*napA*	—	ATGAAGCTTTCATAAAGCTACGACG
aegprom	upstream to *pnapF*	+*	TTCTGCGGCCCAGGCGG
napFHin1	*napF*	—	TATAAGCTTAGGCGTCGCAGCGGGTG
napFHin2	*napF*	+	GTTAAGCTTGTGCAGCCAGCTGTCCG
napDHind1	*napD*	—	TATAAGCTTGGCGTTCAGTTGGGTG
napDHind2	*napD*	+	GTTAAGCTTCACCAGCAGGAAGAGC
napArev	*napA*	—	CCAACAACGGCGCGGGC
erm11	*erm*	+	ATCGGATCCTTAAGAGTGTGTTGATAG
erm22	*erm*	—	ATCGGATCCGATACAAATTCCCCG

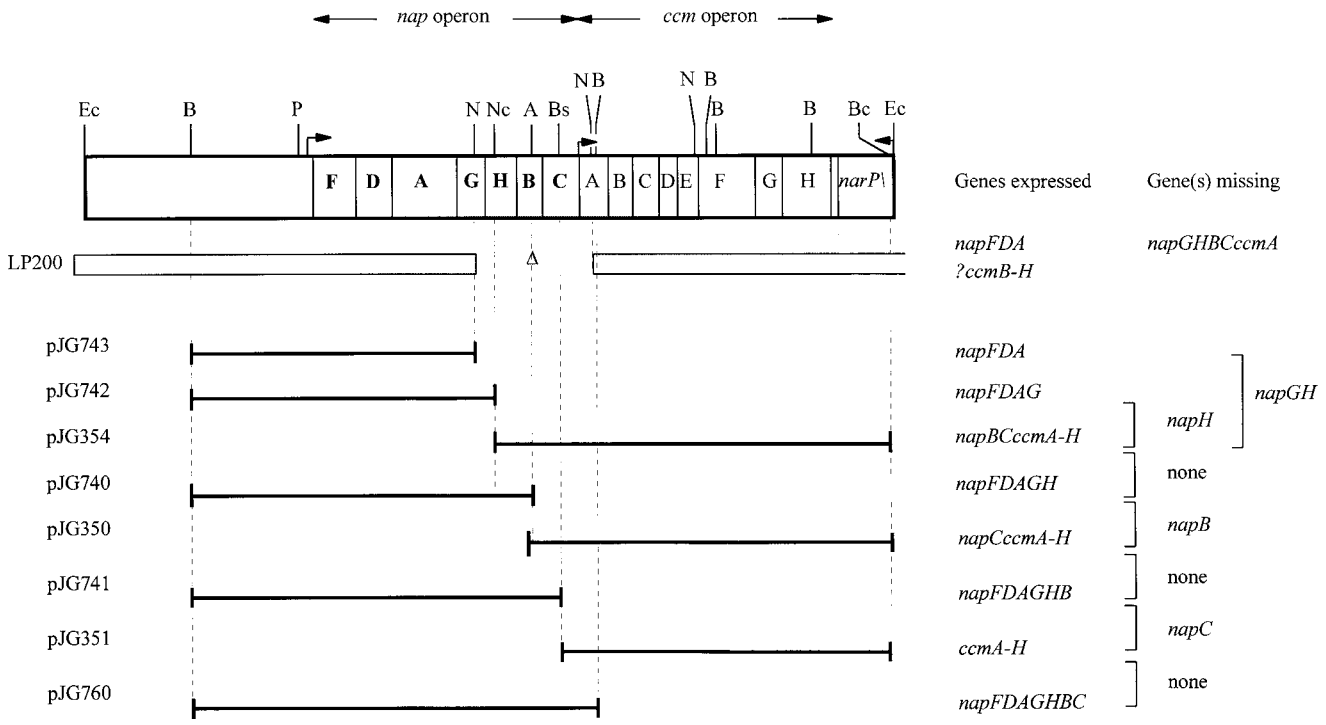

Figure 1 DNA fragments of the *E. coli nap-ccm* region cloned into plasmids used in the present study

The locations of genes and key restriction sites within the 20 kb *Eco*RI fragment of chromosomal DNA in plasmid pLG600, which encodes all of the *nap-ccm* genes as well as the downstream *narP* gene, are shown at the top of the Figure. The vectors are: for the pJG2*xx* series, pUC18; for the pJG3*xx* series, pAA234; and for the pJG7*xx* series, pMAK705 (Table 2). Restriction sites are: B, *Bam*HI; Bc, *Bcl*I; Bs, *Bsp*EI; Ec, *Eco*RI; N, *Nru*I; P, *Pst*I.

Construction of the pJG3*xx* series of plasmids

The vector used to construct the pJG3*xx* series of plasmids was pAA234, a derivative of pBR322 that contains the *galE* promoter preceding a *Sma*I–*Bgl*II–*Hin*dIII cloning site [18]. Plasmid pJG350 expresses the *napC* gene and *ccm* operon from the *galE* promoter [18]. Plasmid pJG354, expressing the *napBC* genes and the *ccm* operon from the vector-encoded *pgalE* promoter, was constructed by cloning the 8.9 kb *Nco*I–*Bcl*I fragment from pJG70 into pAA234. Plasmid pJG355 expresses *napD* from *pgalE*. The *napD* gene was amplified as a 300 bp fragment using primers napDBamHI and napDHin3 with pJG600 as the DNA template.

Construction of the pJG7*xx* series

The cloning vector for the pJG7*xx* series of plasmids was pMAK705. This contains a temperature-sensitive origin of replication preventing replication at 42 °C [34]. Plasmid pJG760 was made by ligating the 10 kb *Bam*HI fragment of pJG600 [17], which encodes the seven *nap* genes under the control of *pnapF*, into pMAK705 that had been restricted with *Bam*HI. Plasmid pJG740 was constructed by digesting pJG760 with *Hin*dIII which cleaves downstream of the 3′ end of the insert. The Klenow fragment was used to fill in the 5′ end and the linear fragment was then digested with *Asc*I, which cuts uniquely within *napB*. The large fragment was circularized to yield a plasmid in which *napFDAGH* are expressed from *pnapF*. Plasmid pJG741 was constructed by digesting pJG760 with *Hin*dIII and filling in with the Klenow fragment. After restriction with *Pst*I, which cuts upstream of *pnapF*, the 6.7 kb *Pst*I *Hin*dIII fragment was replaced by the nested 9 kb *Pst*I *Bsp*EI fragment. In this plasmid, *napFDAGHB* are expressed from *pnapF*. Plasmids pJG742 and pJG743, which express *napFDAG* or *napFDA* respectively, were made by replacing the 6.7 kb *Pst*I–*Hin*dIII fragment of pJG760 with either a 4.5 kb *Pst*I–*Nco*I fragment or a 4.1 kb *Pst*I–*Nru*I fragment.

Construction of pJG744, pJG745 and unmarked chromosomal mutations in *napF* and *napD*

Plasmids pJG744 and pJG745 containing in-frame, unmarked deletions within the *napF* and *napD* genes respectively were constructed by three-way ligation and PCR with pJG600 as template DNA [17]. To make pJG744, fragment F1 was amplified using primers aegprom and napFHind1, which anneal upstream of the *napF* promoter and to the coding strain of *napF* respectively. The PCR product was restricted with *Pst*I and *Hin*dIII. Fragment F2 was made using primers napFHind2 and napArev, which anneal to the template strand of *napF* and the coding strand of *napA*, respectively, and the product was restricted with *Hin*dIII and *Not*I. Fragments F1 and F2 were ligated into pJG760 that had been restricted with *Pst*I and *Not*I. The resulting plasmid, pJG744, contains a deletion of 15 out of the 16 cysteine codons required for the insertion of iron–sulphur clusters into NapF.

Plasmid pJG745 was constructed the same way, except that the primers used to made F1 were aegprom and napDHind1 and for F2 they were NapDHind2 and napArev. NapDHind1 and NapDHind2 anneal to the template and coding strands of *napD* respectively.

The in-frame unmarked deletions in the *napD* and *napF* genes of plasmids pJG744 and pJG645 respectively were transformed

into strain LCB2048 and chloramphenicol-resistant colonies were selected at 30 °C [34]. Five chloramphenicol-resistant transformants were grown overnight at 42 °C and subjected to ampicillin enrichment for isolation of chloramphenicol-sensitive recombinants. PCR was used to screen candidates for those that contained the correct mutation.

Growth of bacteria

Cultures for analysis by SDS/PAGE were grown in 10 ml of minimal salts (MS) medium [35] supplemented with 5% (v/v) Luria–Bertani broth (LB) [36], 40 mM fumarate, 0.4% (v/v) glycerol and 20 mM nitrate. Antibiotics and amino acids were added where necessary. The cultures were incubated at 30 °C without aeration until the attenuance (D_{650}) was between 0.5 and 0.8.

Cultures for nitrate electrode assays or analysis by non-denaturing PAGE were grown at 30 °C in 2-litre conical flasks filled with MS medium supplemented with 50% (v/v) nutrient broth (NB; Oxoid no. 2), 0.4% (v/v) glycerol, 40 mM fumarate and 20 mM nitrate as required.

Use of a nitrate electrode to measure rates of nitrate reduction by physiological substrates

A thermostatically controlled 10 ml reaction chamber was fitted with a non-porous bung through which were inserted an ion-selective nitrate electrode, a temperature probe and steel tubing to allow additions. A nitrate combination electrode (Spectronic Analytical Instruments, Leeds, U.K.) was linked to an ion-meter (model 3345 from Jenway Inc., Princeton, NJ, U.S.A.) and the output, in millimolar nitrate, was recorded by a computer.

For each assay, 0.5–1 ml of the bacterial suspension was diluted to 10 ml with phosphate buffer and equilibrated to 30 °C. Nitrate (2 mM) was added and the assay started by the addition of 0.1 ml of a 2 M solution of electron donor. Each assay was repeated twice for at least two independent bacterial cultures. Unless otherwise stated, the reported rates are the average for these four assays. This assay was optimized for the detection of nitrate reductase A and Nap activity, resulting in a lower limit of detection of 5 nmol of nitrate reduced · min^{-1} · mg of dry bacteria^{-1}.

Isolation of periplasmic proteins from bacterial cultures

Periplasmic proteins were extracted from 2-litre bacterial cultures by treatment with EDTA and lysozyme [37]. After repeated dialysis against 20 vol. of 1 mM Tris/HCl, pH 8.0, at 5 °C, the periplasmic fraction was freeze-dried and resuspended in 500 μl of sterile distilled water.

PAGE and staining methods

For SDS/PAGE, an 18-cm-square-slab apparatus was filled with a 15% (w/v) resolving and a 6% (w/v) stacking polyacrylamide gel. Cultures (1.5 ml samples) were pelleted by centrifugation, resuspended in sample buffer and heated at 100 °C for 10 min prior to loading. After electrophoresis for 17 h at 70 V, proteins with covalently bound haem were identified by their haem peroxidase activity [38].

Concentrated periplasmic proteins were separated on native 7.5% (w/v) polyacrylamide gels. After electrophoresis, gels were placed in a solution of dithionite-reduced Methyl Viologen containing 20 mM nitrate. Nitrate reductase activity was detected as a colourless band against a dark purple background [17].

RESULTS AND DISCUSSION

Detection of periplasmic nitrate reductase activity using physiological electron donors

Strain LCB2048, obtained from Dr. G. Giordano (Laboratoire de Chimie Bacterienne, IFRC Biologie Structurale et Microbiologie, CNRS, Marseilles, France), is a derivative of LCB320 that reportedly contains lesions in the structural genes for both nitrate reductase A and Z [39]. Any residual nitrate reductase activity associated with this strain should therefore be due to Nap. To determine whether this strain retained the ability to reduce nitrate, LCB2048 and its parent, LCB320, were grown anaerobically in the presence of nitrate and washed bacteria were assayed for nitrate reductase activities using the nitrate electrode assay (Table 4). High rates of nitrate reduction were detected, as expected, for strain LCB320, especially with formate as the electron donor. Significant activity was also detected with LCB-2048, especially with formate as the electron donor (105 nmol of nitrate reduced · min^{-1} · mg dry mass^{-1}). Rates of nitrate reduction with glycerol, glucose, lactate, succinate and ethanol were 80, 57, 13, 13 and 2% of the rate with formate respectively.

Strain JCB20480 and its parent, LCB2048, were grown anaerobically in MS medium supplemented with 5% (v/v) LB, glycerol, fumarate, nitrate and trimethylamine-*N*-oxide. The bacteria were pelleted by centrifugation, the proteins separated by SDS/PAGE and stained to detect covalently bound haem. The five expected haem-staining bands present in the wild-type

Table 4 Effects of a non-polar insertion in *napA*, in-frame deletion of *napD* and *napF* and defects in *napB*, *napC*, *napH* and *napGH* on rates of nitrate reduction by Nap after anaerobic growth in the presence of nitrate

Bacteria were grown anaerobically in MS supplemented with 50% (v/v) NB, glycerol, fumarate and nitrate. Rates of nitrate reduction were determined with the growth substrate, glycerol, or the fermentation product, formate, and are given as nmol of nitrate reduced · min^{-1} · mg bacterial dry mass^{-1}. Values are the averages for two independent cultures assayed in duplicate.

Strain	Plasmid(s)	*nap* genes absent	Nitrate reduction by: Glycerol	Formate
Effect of disrupting expression of NRA and NRZ				
LCB320 (parent of LCB2048)		None	192	255
LCB2048		None	155	152
Effect of a non-polar insertion in *napA*				
LCB2048	–	None	155	152
JCB20480	–	*napA*	< 5	< 5
	pJG600	None	173	158
Two-plasmid strategy				
LCB2048	–	None	155	152
LP200	–	*napG-ccmA*	16	15
	pJG740 + pJG350	*napB*	15	16
	pJG741 + pJG350	None	194	166
	pJG741 + pJG351	*napC*	15	14
	pJG760 + pJG351	None	201	165
	pJG742 + pJG354	*napH*	208	125
	pJG743 + pJG354	*NapGH*	130	92
	pJG740 + pJG354	None	231	165
In-frame chromosomal deletions				
LCB2048	–	None	155	152
LP202	–	*napD*	< 5	< 5
	pJG355	None	140	130
LP203	–	*napF*	165	119

strain could also be detected to comparable levels in the mutant. The presence of the 16 kDa NapB and 24 kDa NapC bands in the mutant, JCB20480, indicated that expression of *napB* and *napC* was not significantly affected by the insertion of the erythromycin-resistance cassette and that the *ccm* genes were also being expressed.

No detectable rate of nitrate reduction by the mutant strain, JCB20480, could be detected with formate or glycerol as the electron donor, but the Nap$^-$ phenotype was complemented by transformation with plasmid pJG600, which contains a 20 kb region of DNA encoding the whole of the *nap-ccm* operon [17] (Table 4). The total loss of activity due to insertion of a non-polar cassette into *napA* confirms that all of the activity of strain LCB2048 is due to NapA: it is unlikely that any other nitrate reductase is active under these conditions. This conclusion is supported by the fact that no nitrite could be detected in the growth medium of strain JCB20480.

Two strategies were developed to demonstrate that all eight *ccm* genes downstream of the *nap* operon are essential for the assembly of *c*-type cytochromes in *E. coli* [18]. Both strategies have now been used to determine whether the six *nap* genes other than *napA* are also essential for the assembly of a functional electron transfer chain to NapA.

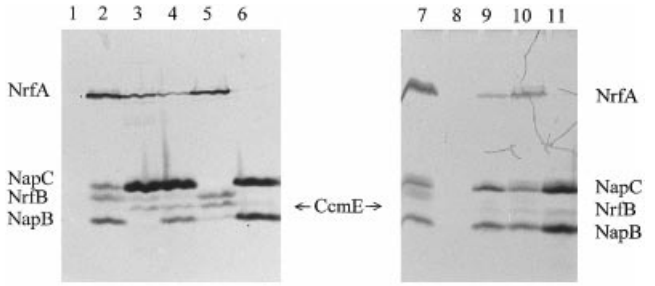

Figure 2 Cytochrome *c* synthesis by strains lacking expression of NapB, NapC, NapH and NapGH

Bacteria were grown in MS medium supplemented with glycerol, fumarate, nitrate and 5% (v/v) LB. After centrifugation, bacteria were lysed in sample buffer, separated by SDS/PAGE and stained for peroxidase activity associated with the covalently bound haem of *c*-type cytochromes [38]. Each track was loaded with the same quantity of biomass. Samples loaded were: lanes 2 and 7, the untransformed strain, LCB2048; lanes 1 and 8, strain LP200 (Δ*napG-ccmA*); lanes 3, 5, 9 and 10, cultures of LP200 transformed with plasmids encoding all genes except *napB* (pJG740 + pJG350), *napC* (pJG741 + pJG351), *napH* (pJG742 + pJG354), and *napGH* (pJG743 + pJG354) respectively. Samples in lanes 4, 6 and 11 were the positive controls for the preceding lanes, as listed in Table 4. Haem-laden CcmE is indicated with an arrow [40,41].

A two-plasmid strategy to generate strains defective in the *c*-type cytochromes NapB and NapC

Strain LP200 is a derivative of LCB2048 with a chromosomal deletion of the *napGHBC* genes and the first of the cytochrome *c* maturation genes, *ccmA*. With either glycerol or formate as the electron donor, strain LP200 retained only about 10% of the activity of the parental strain (Table 4). Plasmid pJG600 complemented the Nap$^-$ phenotype of the mutant strain to a level comparable with, or slightly higher than, that of the wild-type strain. This confirmed that at least one *nap* gene product is essential for Nap activity with physiological electron donors. Any involvement of the Ccm proteins was assumed to be indirect due to their essential requirement in the maturation of the two *c*-type cytochromes, NapB and NapC. Strain LP200 still contains the structural gene for the catalytic subunit and the ability of this strain to reduce nitrate at a very low rate ($\approx$ 15 nmol of nitrate reduced·min^{-1}·mg dry weight^{-1}) might be due to electrons reaching NapA via a physiologically insignificant route. A low concentration of nitrite could also be detected in the growth medium of the mutant, providing further evidence for this proposal.

A two-plasmid strategy was used to determine whether the *c*-type cytochromes, NapB and NapC, are essential for periplasmic nitrate reductase activity. Genes upstream of the gene of interest were cloned under the control of the *napF* promoter into the temperature-sensitive vector, pMAK705 (the pJG7*xx* series of plasmids). Fragments containing the downstream *nap* and *ccm* genes were cloned into the compatible pBR322-based vector, pAA234, and expressed under the control of the *galE* promoter (pJG3*xx* series). Pairs of compatible plasmids were transformed into strain LP200 (Figure 1), and rates of nitrate reduction by the double transformants after anaerobic growth in the presence of both fumarate and nitrate were determined. To generate a derivative of strain LP200 that lacked expression of just *napB*, plasmids pJG740 and pJG350 were co-transformed into strain LP200 (Figure 1). Similarly, plasmids pJG741 and pJG351 were co-transformed into strain LP200 to generate a strain lacking expression of just *napC*. LP200 was also transformed either with pJG760 and pJG351, or with pJG741 and pJG350, to give positive control strains that express all of the *nap-ccm* genes and to ensure that any negative results were not artefacts due to the use of multi-copy plasmids. The *c*-type cytochromes synthesized by strains LCB2048, LP200 and the double transformants during anaerobic growth in the presence of nitrate were determined by SDS/PAGE (Figure 2). As expected, none of the *c*-type cytochromes synthesized by strain LCB2048 were made by the mutant, LP200, which lacks expression of *ccmA* (Track 1). No NapB or NapC protein was detected in the transformants of LP200 which lack expression of either *napB* or *napC* respectively (lanes 3 and 5). Restoration of expression resulted in the overexpression of both NapB and NapC to similar levels, but virtually no NrfA or NrfB could be detected (lanes 4 and 6).

After anaerobic growth in the presence of nitrate, only a low level of formate- or glycerol-dependent Nap activity, similar to that detected in strain LP200, was detected with the nitrate electrode in strains lacking expression of either NapB or NapC (Table 4). In contrast, activities of the double transformants that served as the positive controls were similar to those of the parental strain, LCB2048. These results established that NapB and NapC are essential for Nap activity with physiological electron donors.

An essential role for NapD in nitrate reduction by Nap

The alternative strategy of constructing strain LP202 with an in-frame deletion in *napD* was used to determine whether NapD is essential for the expression of a Nap activity. After anaerobic growth in the presence of nitrate, no nitrate reduction could be detected by strain LP202 with either glycerol or formate as the electron donor (Table 4). In addition, no nitrite could be detected in the growth medium. Transformation of strain LP202 with pJG355, a plasmid that expresses *napD* from the *galE* promoter, resulted in complementation of the Nap$^-$ phenotype to near wild-type levels. These results confirm that NapD is essential for periplasmic nitrate reduction by physiological electron donors.

The lack of structural and functional motifs in the NapD amino acid sequence has hindered the assignment of a role to NapD in periplasmic nitrate reduction, but it is unlikely to have a function in electron transfer. To investigate whether NapD might have a role in the maturation of the molybdoenzyme, NapA [24], strains LCB2048, LP202 and LP202 transformed

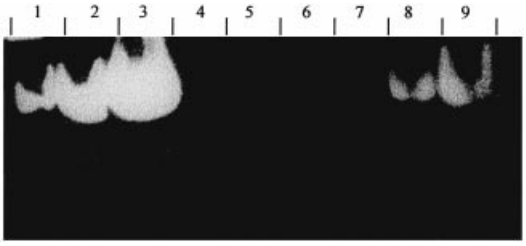

Figure 3 Effects of deleting *napD* on Methyl Viologen-dependent NapA activity

Bacteria were grown anaerobically in MS medium supplemented with glycerol, fumarate, nitrate and 50% (v/v) NB. Periplasmic proteins were separated by non-denaturing PAGE and stained for Methyl Viologen-dependent nitrate reductase activity in the presence of nitrate. For each culture, 50, 150 and 450 μg of sample were loaded on to three successive lanes. Lanes 1–3, LP200.pJG355; lanes 4–6, LP200; and lanes 7–9, LCB2048.

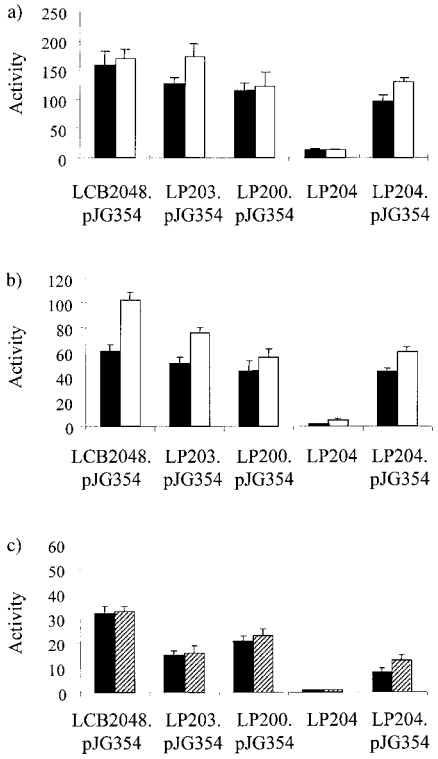

Figure 4 Effects of loss of NapF and NapFGH expression on nitrate reduction by Nap

Bacteria were grown anaerobically in MS medium supplemented with 50% (v/v) NB and: (**a**) glycerol, fumarate and nitrate; (**b**) glycerol and fumarate; and (**c**) glucose. Rates of nitrate reduction were determined with formate (black bars), glycerol (white bars) or glucose (hatched bars) as electron donors. Error bars indicate the range of activities obtained for two or more replicate experiments with independently grown cultures on different days. Rates are given as nmol of nitrate reduced $\cdot$ min^{-1} $\cdot$ mg of bacterial dry mass^{-1}. Values given are the averages for at least two independent cultures assayed in duplicate. Note the different scales in the three parts of the Figure.

with the *napD*$^+$ plasmid, pJG355, were grown anaerobically in the presence of nitrate. Bacteria were harvested and periplasmic proteins were isolated by treatment with EDTA and lysozyme. Increasing amounts of protein for each strain were separated by non-denaturing PAGE and stained for Methyl Viologen-dependent NapA activity (Figure 3). After 15 min staining, bands of activity could be detected for the wild-type strain, LCB2048 (lanes 7–9), and the mutant transformed with pJG355 (lanes 1–3). No band was detected for LP202, even in the lane that had been loaded with 450 μg of periplasmic protein (lanes 4–6). These results confirm that NapD is required for the synthesis of a functional NapA molybdoenzyme. Although the bands of Nap activity obtained for the mutant strain transformed with plasmid pJG355 were much brighter than those for the parental strain LCB2048, the rates of nitrate reduction by the transformant were not significantly higher than those of LCB2048.

In conclusion, NapD is required for the synthesis of a functional NapA protein. However, these results do not establish whether the absence of NapD leads to the synthesis of an inactive form of the enzyme or to the total loss of NapA biosynthesis.

Effect of deleting *napF*, *napGH* or *napFGH* on Nap activity

The formate-dependent rate of nitrate reduction by strain LP203, with an in-frame deletion in the *napF* gene, was 85% of that of strain LCB2048. The activity with glycerol as the electron donor was equivalent to that of the parental strain (Table 4), so unlike *R. sphaeroides* DSM158 [26], NapF is not essential for electron transfer to NapA.

Plasmid pJG354 complemented the Nap$^-$ phenotype of strain LP200 by restoring expression of the essential *napBC* and *ccmA–ccmH* genes to generate a strain lacking expression of only *napG* and *H*. Approx. 76% of the formate-dependent and 72% of the glycerol-dependent activity was retained when compared with the parental strain, LCB2048.pJG354 (Figure 4). Furthermore, rates of nitrate reduction by strain LCB2048.pJG354, which expresses all seven *nap* genes, gave rates of 158 and 169 units with formate and glycerol as electron donors respectively. These values were not significantly different from the rates measured in the absence of the plasmid (152 and 155 units respectively; Table 4).

Strain LP204, a derivative of LP200 with an additional deletion in the *napF* gene, had the expected Nap$^-$ phenotype (Figure 4). Transformation with pJG354 generated a Δ*napFGH* strain that retained 60% of the formate-dependent and 76% of the glycerol-dependent Nap activity of LCB2048.pJG354 (Figure 4). Therefore, none of the three iron–sulphur proteins are essential for electron transfer from the quinone pool to NapA during nitrate respiration.

Essentially similar results were obtained using the two-compatible-plasmid strategy. Strain LP200 was transformed with plasmids pJG742 and pJG354 (Figure 1) to restore expression of all the missing and unexpressed genes except *napH*, with plasmids pJG743 and pJG354 to generate a strain that lacked expression of both *napG* and *napH*, and with pJG740 and pJG354 to provide the isogenic positive control (Figure 1). The double transformant lacking expression of *napH* retained between 75 and 90% of the activity of the positive control, depending upon the electron donor used in the assay (Table 4). The further loss of NapG caused both of these values to decrease to around 55%, confirming that neither NapG nor NapH is essential for electron transfer to the catalytic subunit, although it is possible that their absence causes a reduction in the electron flow to NapA.

Are all of the *nap* genes essential for Nap activity during anaerobic growth by fumarate respiration or glucose fermentation?

The periplasmic location of Nap means that any requirement for nitrate uptake is by-passed. A possible role for Nap might therefore be to allow rapid adaptation to nitrate respiration from

Table 5 Effects of deleting *napB*, *napC*, *napG* and *napGH* on rates of nitrate reduction by Nap after growth by fumarate respiration or glucose fermentation

Bacteria were grown anaerobically in MS supplemented with 50% (v/v) NB and either glycerol and fumarate or glucose. Rates of nitrate reduction were determined with formate, glycerol or glucose as electron donors using the nitrate electrode assay. Rates are given as nmol of nitrate reduced · min^{-1} · mg of bacterial dry $mass^{-1}$. Values given are the averages for at least two independent cultures assayed in duplicate.

			Rates of nitrate reduction after growth by:			
			Fumarate respiration		Glucose fermentation	
Strain	Plasmids	*nap* genes absent	Formate	Glycerol	Formate	Glycerol
LCB2048	–	None	95	54	30	28
LP200	–	*napG-ccmA*	6	< 5	< 5	< 5
	pJG740 + pJG350	*napB*	8	< 5	< 5	< 5
	pJG741 + pJG350	None	189	160	92	89
	pJG741 + pJG351	*napC*	6	< 5	< 5	< 5
	pJG760 + pJG351	None	200	180	87	98
	pJG742 + pJG354	*napH*	133	96	54	61
	pJG743 + pJG354	*napGH*	80	70	21	31
	pJG740 + pJG354	none	214	186	134	125

other, energetically less favourable, modes of growth. If so, it must be expressed during anaerobic growth in the absence of nitrate and have the potential to reduce nitrate immediately it appears.

Strain LCB2048 maintained significant rates of nitrate reduction after anaerobic growth in the presence of fumarate (Table 5). Nitrate reduction was also detected after anaerobic, fermentative growth in the presence of glucose, with both formate and glucose proving to be effective electron donors to Nap (Table 5). The level of Nap activity after anaerobic growth with fumarate was lower than that seen after anaerobic growth in the presence of nitrate and lowest after fermentative growth. Only 18% of the formate-dependent Nap activity was detected after anaerobic, fermentative growth compared with nitrate respiration (compare Tables 4 and 5). Nevertheless, sufficient Nap was synthesized for the bacteria to be able to reduce nitrate immediately it becomes available. Strain LP200 maintained a consistent Nap^- phenotype regardless of the growth medium, indicating that at least one of the *napGHBC* genes are always essential for electron transfer to the catalytic subunit.

The possibility was investigated that NapF alone, or at least one of the iron–sulphur proteins, F, G or H, are required for Nap-dependent nitrate reduction after anaerobic growth by fumarate respiration, or after fermentative growth. For these experiments, strains LP200, LP203 and LP204 were transformed with pJG354 and grown anaerobically in minimal medium supplemented with either glycerol and fumarate as the carbon and energy source, or with glucose alone. Strain LCB2048.pJG354 provided the positive control. After growth by fumarate respiration, rates of formate-dependent and glycerol-dependent nitrate reduction by this strain were 61 units and 102 units (Figure 4b), comparable with those obtained for the untransformed strain (54 and 95 units respectively). The Δ*napF* strain, LP203.pJG354, retained between 75 and 85% of the activity of the control strain, depending on the electron donor (Figure 4b), so NapF is not essential for electron transfer to the catalytic subunit after growth in the presence of fumarate.

Strain LP200.pJG354 lacks expression of *napG* and *napH*. This strain retained approx. 77 and 57% of the formate- and glycerol-dependent activities respectively compared with the parental strain, LCB2048.pJG354 (Figure 4b). Similar results were obtained with strain LP204.pJG354, which lacks *napF*, *G* and *H* genes. Rates of nitrate reduction by this strain with formate and glycerol as the electron donors were approx. 75 and 62% of the corresponding rates of the parental strain, LCB-2048.pJG354 (Figure 4b).

Essentially similar results were obtained after fermentative growth with glucose alone. Strain LP203.pJG354, which contains a chromosomal deletion in *napF*, retained 47% of the formate-dependent and 49% of the glucose-dependent activity of the parental strain, LCB2048.pJG354, establishing that NapF is not essential for electron transfer to NapA after fermentative growth (Figure 4c). Strain LP200.pJG354 (deleted for *napGH*) retained 66% of the formate-dependent and 70% of the glucose-dependent activity of the control strain. In comparison, the Δ*napGH* strain constructed using the two compatible plasmid strategy retained between 25 and 16% of the activity of the control strain with formate and glucose respectively (Table 5). Strain LP-204.pJG354 (lacking *napFGH*) retained 25% of the formate-dependent and 39% of the glucose-dependent activity of the parental strain (Figure 4c). These results show that a strain deleted for all three *nap*-encoded iron–sulphur proteins is still able to support a significant rate of nitrate reduction, even after fermentative growth. In conclusion, none of the iron–sulphur proteins, NapF, NapG and NapH, are essential for electron transfer to NapA after anaerobic growth with fumarate or during fermentative growth. However, there was a significantly greater decrease in the Nap activity of the strain lacking *napFGH* after growth on glucose than after growth on either nitrate or fumarate. It is therefore possible that these three proteins have a role in the adaptation to nitrate respiration from fermentation.

Strains lacking expression of *napB* and *napC*, constructed using the two compatible plasmid strategy, were also grown anaerobically in MS medium supplemented with 50% (v/v) NB, glycerol and fumarate, or with glucose as a fermentable carbon source. The phenotype of the Δ*napB* strain was Nap^- (Table 5). This was complemented to Nap^+ upon restoration of *napB* expression. An analogous result was obtained for the strain deleted for *napC*. The rates of nitrate reduction by the two positive control strains [LP200.pJG741.pJG350 ($NapB^+$) and LP200.pJG760.pJG351 ($NapC^+$)] after anaerobic growth with fumarate were very similar to the rates obtained after anaerobic growth with nitrate (Table 5). It was concluded that both *c*-type cytochromes are essential for periplasmic nitrate reduction after fermentative growth or growth with fumarate as the terminal electron acceptor.

Do the Nap proteins form a complex in the periplasm?

The amount of NapB made by the strain lacking expression of NapC was significantly lower than that synthesized by the parental strain, LCB2048 (Figure 2, lanes 1 and 5). This was surprising, because the former contains multiple copies of both the *napB* structural gene and the *ccm* genes. Restoration of *napC* expression resulted in the overexpression of NapB and NapC to similar levels, but virtually no NrfA or NrfB could be detected (lane 6).

To investigate whether the loss of NapH or both NapG and NapH might affect cytochrome *c* synthesis, LCB2048, LP200 and the three double transformants (including the positive control) were grown anaerobically in MS medium supplemented with 5% (v/v) LB, glycerol, fumarate and nitrate. The cell

proteins were separated by SDS/PAGE and stained for covalently bound haem (Figure 2). All transformants were able to synthesize NrfA, albeit to markedly different levels (lanes 9–11). Overexpression of the *ccm* genes resulted in the virtual loss of NrfB and the appearance of the haem-binding protein, CcmE. Levels of NapB and NapC made by the Δ*napH* and Δ*napGH* strains were again lower than those synthesized by the positive control strain (compare lanes 9 and 10 with lane 11). It is unlikely that expression of *napB* and *napC* would differ significantly from one transformant to another, as the *napG* and *H* genes, when present, were always expressed from a plasmid different from that encoding *napBC*.

The decreased synthesis of NapB and NapC by strains lacking expression of *napC*, *napH* or *napGH* suggests that the absence of one or more Nap genes causes two cytochromes to be more prone to proteolysis. This might also be true for NapA. Furthermore, decreased synthesis of the essential NapB and NapC proteins could account for the lower Nap activity measured for the Δ*napH* and Δ*napGH* strains. These results suggest that the Nap proteins might form a functional complex that is attached, albeit loosely, to the periplasmic face of the cytoplasmic membrane. NapH and NapC are both membrane-bound proteins, and the formation of a stable complex between them and the soluble NapB, NapG and NapA proteins would be advantageous to the bacteria as it would facilitate efficient electron transfer from the quinone pool to the catalytic site.

The variable amounts of NrfA and NrfB synthesized by strains overexpressing different combinations of *nap* genes suggests that the apocytochromes compete for haem attachment. In general, strains that over-expressed the NapB or NapC structural genes synthesized less NrfA and NrfB (compare lanes 2, 3 and 6 with lane 5). Surprisingly, NrfA and NrfB synthesis did not increase significantly in the strain that lacked expression of *napC*, even though it overexpressed the *ccm* genes and NapB levels were very low (Figure 2; compare lanes 1 and 5).

Conclusions

The data presented clearly establish that the products of the *napD*, *A*, *B* and *C* genes are all essential for nitrate reduction by the periplasmic nitrate reductase of *E. coli*. Furthermore, evidence is presented that the cytoplasmic NapD plays a currently undefined role in the post-translational assembly of a functional molybdopolypeptide, NapA. Although NapF, NapG and NapH all contribute to the maximum rate of nitrate reduction in the periplasm, their roles remain unknown. We have eliminated the possibility that they are essential for coupling electron transfer from physiological substrates to NapA during fermentative growth, but the possibility remains that they preferentially couple electron transfer from either menaquinone or, less likely, ubiquinone, to the essential Nap polypeptides NapC, NapB and NapA. The experiments described failed to reveal a physiological role of Nap in enteric bacteria: this is the subject of the accompanying paper [43] .

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