# Competition between *Escherichia coli* strains expressing either a periplasmic or a membrane-bound nitrate reductase: does Nap confer a selective advantage during nitrate-limited growth?

Laura C. POTTER, Paul MILLINGTON, Lesley GRIFFITHS, Gavin H. THOMAS[1] and Jeffrey A. COLE[2]
School of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

The physiological role of the periplasmic nitrate reductase, Nap, one of the three nitrate reductases synthesized by *Escherichia coli* K-12, has been investigated. A series of double mutants that express only one nitrate reductase were grown anaerobically in batch cultures with glycerol as the non-fermentable carbon source and nitrate as the terminal electron acceptor. Only the strain expressing nitrate reductase A grew rapidly under these conditions. Introduction of a *narL* mutation severely decreased the growth rate of the nitrate reductase A strain, but enhanced the growth of the Nap$^+$ strain. The ability to use nitrate as a terminal electron acceptor for anaerobic growth is therefore regulated primarily by the NarL protein at the level of transcription. Furthermore, the strain expressing nitrate reductase A had a substantial selective advantage in competition with the strain expressing only Nap during nitrate-sufficient continuous culture. However, the strain expressing Nap was preferentially selected during nitrate-limited continuous growth. The saturation constants for nitrate for the two strains (which numerically are equal to the nitrate concentrations at half of the maximum specific growth rate and therefore reflect the relative affinities for nitrate) were estimated using the integrated Monod equation to be 15 and 50 $\mu$M for Nap and nitrate reductase A respectively. This difference is sufficient to explain the selective advantage of the Nap$^+$ strain during nitrate-limited growth. It is concluded that one physiological role of the periplasmic nitrate reductase of enteric bacteria is to enable bacteria to scavenge nitrate in nitrate-limited environments.

Key words: chemostat, dual nitrate reductases.

## INTRODUCTION

The role of membrane-associated nitrate reductases in energy conservation during anaerobic bacterial growth has been known for many years [1]. Only more recently, however, has it been appreciated that some Gram-negative bacteria also synthesize alternative, soluble nitrate reductases [2,3], and McEwan et al. were the first to demonstrate their periplasmic location with the biochemical characterization of a periplasmic nitrate reductase (Nap) from the phototrophic bacterium *Rhodobacter capsulatus*. [4]. The first genetic studies based upon the *nap* operon of *Ralstonia* (formerly *Alcaligenes*) *eutrophus* [5] were soon followed by sequencing of the *nap* operons from *Thiosphaera pantotropha* [6] and subsequently many other bacteria, including *Escherichia coli* [7], *Rhodobacter sphaeroides* [8] and a rhizobium that was initially called *Pseudomonas* G-179 [9,10]. All of the operons so far identified include four genes in the order *napDABC*, but between one and three out of five other genes occur in different combinations among the various types of bacteria. This diversity might imply that the physiological roles of Nap differ between species, but whether there is any correlation between the different components synthesized and the physiological roles of Nap in different types of bacteria remains to be established.

In the absence of oxygen, *E. 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, two attached to the cytoplasmic membrane and the third located in the periplasm, as summarized in the accompanying paper [11]. Unlike the *nap* operons of *T. pantotropha* and *R. eutrophus*, the *E. coli napFDAGHBC* operon is transcribed only during anaerobic growth [12–14]. Similar operons in *Salmonella typhimurium* and *Haemophilus influenzae* have been revealed by genome sequencing. The availability of *E. coli* strains that express only one of the three types of nitrate reductase would provide an excellent model system for comparing the physiological roles of the different nitrate reductases in enteric bacteria. The construction and anaerobic growth characteristics of such strains are now described. We have investigated whether Nap can support anaerobic growth of *E. coli* on a non-fermentable carbon source and, if so, whether there are growth conditions in which the presence of Nap provides a selective advantage relative to bacteria that express only nitrate reductase A. Finally, the biochemical basis for the different physiological roles of Nap and nitrate reductase A is reported.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and oligonucleotides

Strains used were the derivatives of *E. coli* K-12 listed in Table 1. Agar plates were kept at 4 °C for several weeks for short-term storage of bacteria. For longer-term storage, 0.75 ml of an overnight liquid culture was mixed with 0.25 ml of a sterile solution of 60 % (v/v) glycerol in Luria–Bertani broth (LB; [15]) and stored at −70 °C. Stock solutions of erythromycin and

Abbreviations used: LB, Luria–Bertani broth; MS, minimal salts.

[1] Present address: Nitrogen Fixation Laboratory, John Innes Centre, Norwich NR4 7UH, U.K.
[2] To whom correspondence should be addressed (e-mail j.a.cole@bham.ac.uk).

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

| Strain | Description | Reference or source |
|---|---|---|
| JC7623 | *recB15 recC22 sbcB15* | [34] |
| JCB4011 | RK4353 Δ*napA-B narZ*::Ω | The present study |
| JCB4012 | JCB4011 *narL*::Tn10 | The present study |
| JCB4021 | RK4353 *narG*::ery *narZ*::Ω | The present study |
| JCB4022 | RK4353 *narG*::ery Δ*napA-B* | The present study |
| JCB4023 | JCB4022 *narZ*::Ω | The present study |
| JCB4024 | JCB4021 *narL*::Tn10 | The present study |
| JCB4031 | RK4353 Δ*narG-I narZ*::Ω | The present study |
| JCB7123 | *narL*::Tn10 derivative of JCB7120 | [35] |
| RK4353 | Parental strain; prototrophic wild-type | V. Stewart* |

* Department of Microbiology, University of California, Davis, CA, U.S.A.

**Table 2 Plasmids used in the present study**

| Plasmid | Description | Reference or source |
|---|---|---|
| pBR322 | 4.36 kb Amp® Tet® medium-copy-number cloning vector with ColE1 origin of replication | [36] |
| pHP45Ω | 4.3 kb Amp® plasmid containing the 2 kb Ω interposon conferring Spec® and Str® | [37] |
| pJG747 | Derivative of pJG760 containing a deletion from the *Bcl*I site of *napA* to the *Asc*I site of *napB* | The present study |
| pJG760 | 10 kb fragment encoding the *napFDAGHBC* operon ligated into pMAK705 | [11] |
| pLP100 | 4 kb PCR fragment containing part of the *narZ* and *narY* genes ligated into pBR322 | The present study |
| pLP101 | Ω fragment from pHP45Ω ligated into pLP100 | The present study |
| pMAK705 | 5.51 kb Chlor®, temperature-sensitive, low-copy-number vector compatible with pBR322 | [16] |
| pSG200 | 4.4 kb fragment containing part of the *narG* and *narY* genes in pUC18 | The present study |
| pSG201 | 1 kb Ery® cassette ligated into unique *Nsi*I site of pSG200 | The present study |
| pSG700 | 4 kb fragment containing the 5′ end of *narG* fused to the 3′ end of *narI* in pMAK705 | The present study |
| pVA50 | 10.9 kb plasmid containing the *narGHJ* genes regulated by *ptac* | [17] |
| pUC18 | 2.6 kb Amp® high-copy-number vector | Pharmacia |

**Table 3 Oligonucleotide primers used in the present study**

+ refers to coding strand, − refers to complementary strand and * denotes that assignment is with respect to the direction of transcription from *pnarG*. Engineered restriction sites are underlined.

| Primer | Location | Strand | Sequence (5′ → 3′) |
|---|---|---|---|
| NarGBamHI | *pnarG* | + | TATGGATCCCACAGGAGAAAACCG |
| NarGAscI | *narG* | − | TATTGGCGCGCCAGCGATAGTCAGCG |
| NarIAscI | *narI* | + | TATTGGCGCGCCAAGTGAAACAGAAAATGGC |
| BeyondI | downstream to *narI* | −* | TATTATCTGCAGTATGCGGGTATTAACGCC |
| NarZHindIII | *narZ* | + | TATAAGCTTCACGGACAGGTGATGC |
| NarYBamHI | *narY* | − | TATGGATCCGCCCCCAGATCACTTTG |

spectinomycin were added to liquid or molten agar medium to give final concentrations of 150 $\mu g \cdot ml^{-1}$ and 50 $\mu g \cdot ml^{-1}$ respectively. Plasmids and oligonucleotides are listed in Tables 2 and 3 respectively.

## Construction of unmarked, chromosomal deletions within the *narG* and *nap* loci

Unmarked, non-polar deletions within the *narG* and *nap* loci were constructed by exploiting the temperature-sensitive origin of replication of plasmid pMAK705 to transfer mutations on to the bacterial chromosome [16].

Plasmid pSG700 containing the 5′ end of *narG* translationally fused to the 3′ end of *narI* was constructed by a three-way ligation of two, 2 kb PCR products (F1 and F2) and the vector pMAK705. Chromosomal DNA from strain JCB4011 was used as template. F1 was amplified using primers NarGBamHI and NarGAscI, which anneal upstream of *pnarG* and to *narG*. F2 was amplified using primers NarIAscI and BeyondI, which anneal to *narI* and just downstream of *narI*. The Δ*narG-I* mutation was transferred on to the chromosome as described previously (the accompanying paper [11], and [16]). Candidates were screened for loss of Methyl Viologen-dependent nitrate reductase activity.

Plasmid pJG760 contains the whole of the *nap* operon cloned into the vector pMAK705 with the *napF* promoter regulating gene expression (the accompanying paper [11]). Plasmid pJG747 is a derivative of pJG760 that contains an unmarked deletion extending from *napA* to *napB*. This plasmid was constructed by digesting pJG760 with *Bcl*I and *Asc*I, which restrict uniquely within the middle of the *napA* and *napB* genes respectively. The

large fragment was circularized to generate a plasmid that only contains *napFD* and *C*. Plasmid pJG747 was transformed into recipient strains and the Δ*napA-B* mutation was transferred on to the chromosome (the accompayning paper [11], and [16]). PCR was used to screen for correct candidates, and further tests showed that the mutation was non-polar on *napC* and *ccmA-H* gene expression.

### Insertional mutation of the *narG* and *narZ* genes with antibiotic resistance cassettes

Interruption of *narG* and *narZ* expression was achieved by insertion of DNA cassettes that encode resistance to erythromycin and spectinomycin respectively. The *narG*::*ery* mutation was constructed by subcloning the 4.4 kb *Pvu*II fragment from plasmid pVA50 [17] into pUC18, which had been digested with *Eco*RI and *Hin*dIII and end-filled with the Klenow fragment of DNA polymerase. The resulting plasmid, pSG200, contained most of *narG* and the first half of *narH*. A 1 kb cassette conferring erythromycin resistance was generated by PCR (the accompanying paper [11]), restricted at each end with *Bam*HI and end-filled with the Klenow fragment. This cassette was ligated into the unique *Nsi*I site within the middle of *narG* to generate plasmid pSG201. This plasmid was linearized and transformed into the *recB recC sbcB* strain JC7623 with selection for growth in the presence of erythromycin. The mutation was transferred by P1 transduction into recipient strains and correct candidates were isolated by loss of Methyl Viologen-dependent nitrate reductase activity.

The *narZ*::Ω mutation was constructed by amplifying a 4 kb region containing most of *narZ* and the 5′ end of *narY* from the chromosome of strain RK4353 using primers narZHindIII and narYBamHI. The PCR product was restricted at the primer-encoded *Bam*HI and *Hin*dIII sites and ligated into pBR322 that had been digested with the same enzyme to generate plasmid pLP100. The Ω interposon was isolated from plasmid pHP45Ω [18] on *Bam*HI 5′ overhangs and ligated into pLP100 that had been digested at the unique *Bcl*I site within *narZ*. The resulting plasmid, pLP101, was linearized and transformed into strain JC7623 with selection for growth in the presence of spectinomycin. The mutation was transferred by P1 transduction into recipient strains and PCR was used to screen for correct candidates.

### Growth of bacteria

A defined medium (GM medium), modified from a recipe obtained from Profesor J. Weiner (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada), was used for the growth experiments. This contained: casein acid hydrolysate (Difco), 0.05 %; thiamine hydrochloride, 0.002 % (w/v); $K_2HPO_4$, 10.5 % (w/v); $KH_2PO_4$, 5.4 % (w/v); and $(NH_4)_2SO_4$, 2 % (w/v). *E. coli* sulphur-free salts, sodium selenate and ammonium molybdate were added as described in [19]. GM medium was supplemented with 0.8 % (v/v) glycerol and 20 mM nitrate as required.

The feed medium for the preliminary continuous-culture experiments was minimal salts (MS) medium [19] supplemented with 50 % (v/v) nutrient broth (NB; Oxoid no. 2) and, to ensure that both strains in a mixed culture were able to grow anaerobically under the conditions imposed, 40 mM fumarate. Glycerol was supplied as a non-fermentable carbon and energy source at a concentration of 0.1 % (v/v) for glycerol-limited and 0.4 % (v/v) for glycerol-sufficient cultures. Nitrate was added to a final concentration of 5 mM for nitrate-limited cultures and 30 mM when glycerol was the growth-limiting nutrient. In the final series of continuous-culture experiments, GM medium was used supplemented with either 5 mM nitrate and 0.4 % (v/v) glycerol for nitrate-limited cultures; or with 50 mM nitrate and 0.1 % (v/v) glycerol when nitrate was in excess. No fumarate was added to these cultures.

Starter cultures for the fermenter experiments were grown anaerobically in batch cultures. The medium used was the same as that used in the fermenter, except that nitrate was omitted, 40 mM fumarate was always present and the glycerol concentration was 0.4 % (v/v).

### Growth of bacteria in a continuous fermenter

A 2-litre MBR Mini-Bioreactor was used. The working volume was varied in different experiments between 1.5 and 2 litres by adjusting the weir. Feed medium was prepared as 10-litre batches and supplied via a peristaltic pump at dilution rates between 0.02 and 0.1, as stated. The culture was stirred at 500 rev./min to maintain good mixing, but was not aerated. The pH was monitored, but no adjustment was required during an experiment. Samples of 20 ml were withdrawn aseptically each day and checked for purity microscopically (Gram stain) and by plating on to nutrient agar. Serial dilutions were also plated on to nutrient agar to obtain well-isolated colonies that were checked by replica plating for the retention of spectinomycin resistance associated with the *narZYWV* mutation. The attenuance ($D_{650}$) was measured and the biomass calculated using the experimentally determined conversion factor that a $D_{650}$ of 1.0 corresponds to 0.4 g of biomass·litre$^{-1}$. These samples were also used to measure concentrations of nitrate and nitrite and rates of nitrate reduction by the artificial electron donor, reduced Methyl Viologen. When a steady state had been achieved, 500 ml samples were withdrawn aseptically, the bacteria were harvested by centrifugation, and rates of formate- and glycerol-dependent nitrate reduction were determined by the nitrate electrode method (the accompanying paper [11]).

### Competition between strains expressing either Nap or nitrate reductase A

To establish mixed cultures of two strains, bacteria were first grown as anaerobic batch cultures in aspirator bottles. Equal volumes of each culture were added to the fermenter at the start of the experiment. The ratio of the colony-forming units of each type was determined by plating dilutions on to nutrient agar and incubation at 37 °C overnight. The resulting colonies were then replica plated on to nutrient agar supplemented with antibiotics to distinguish between the two types. When the antibiotic-resistance determinants were identical in the two strains, diluted samples were plated to obtain well-isolated single colonies, which were grown anaerobically in the presence of nitrate as pure cultures in test tubes and assayed for Methyl Viologen-dependent nitrate reductase activity.

### Determination of the concentration of nitrate in the presence of nitrite

In some experiments it was necessary to confirm that excess nitrate was available in the chemostat. High concentrations of nitrite accumulated in these cultures, which make it difficult to use the nitrate electrode to measure the nitrate concentration because the electrode responds to both nitrate and weakly to nitrite. This problem was overcome by exploiting a triple mutant, strain JCB4023, devoid of nitrate reductase activity. The mutant, which expresses both the Nir and Nrf pathways for nitrite reduction, was grown anaerobically in the presence of 2.5 mM

nitrite, harvested, washed and resuspended to a biomass concentration of about 3 mg of dry mass/ml in MS supplemented with 0.4 % (v/v) glycerol. This suspension was mixed with an equal volume of a 10-fold-diluted sample of the culture medium from which bacteria had been removed by centrifugation. The mixture was incubated at 37 °C for 1 h and, when the last traces of nitrite had been reduced, centrifuged to pellet the bacteria. The presence of nitrate in the supernatant was then confirmed using the nitrate electrode.

### Use of the Methyl Viologen assay to determine nitrate reductase activities and the ratio of bacteria expressing the periplasmic nitrate reductase and nitrate reductase A

Rates of nitrate reduction by the artificial electron donor, reduced Methyl Viologen, were determined using a previously described method [1].

Samples of two-component cultures of bacteria carrying identical antibiotic-resistance determinants were diluted $10^5$- or $10^6$-fold and plated on to nutrient agar and grown overnight at 37 °C. A total of 24 well-isolated colonies were transferred into 1 ml of LB in 24 test tubes. After overnight growth at 37 °C without aeration, 9 ml of prewarmed MS supplemented with 0.4 % (w/v) glycerol, 40 mM fumarate, 5 % (v/v) LB, 5 $\mu g \cdot ml^{-1}$ thiamine and 20 mM nitrate was added and cultures were incubated at 37 °C until the $D_{650}$ had increased to above 0.5. The bacteria were sedimented by centrifugation (5000 ***g*** for 5 min) and resuspended in 4 ml of 50 mM phosphate, pH 7.4. This suspension (50 $\mu$l) was incubated with reduced Methyl Viologen and nitrate as in the standard assay for Methyl Viologen-dependent nitrate reductase activity. After 5 min at 30 °C, each assay was terminated by vortex-mixing for 10 s, and 1 ml each of sulphanilamide and naphthylethylene diamine dihydrochloride reagents were added with thorough mixing. A dark pink colour was obtained immediately with cultures of strains expressing nitrate reductase A, but those for cultures expressing only Nap were pale pink or almost colourless. Control experiments with alternative double mutants with different antibiotic resistance determinants established that this is a reliable method for distinguishing between bacteria that express only one nitrate reductase, A or Nap. Activities of the *nap*$^+$ clones were $13 \pm 6$ units compared with a mean value of $252 \pm 8$ units for bacteria from the *narG*$^+$ colonies. Some samples from the chemostat were assayed independently by different people and on different days: their data agreed within the variation expected statistically from random sampling of the colonies.

### Rates of nitrate reduction by glycerol and formate and estimation of the saturation constant for nitrate

Rates of nitrate reduction by physiological substrates such as formate and glycerol were determined exactly as described in the accompanying paper [11]. The apparent saturation constants, $K_s$, for nitrate of bacteria expressing either only nitrate reductase A or Nap were determined from progress curves of reactions in the nitrate electrode chamber. Data from several independent assays were fitted to the integrated form of the Monod equation (which is similar to the Michaelis–Menton equation) as described by Wharton and Eisenthal [20]. The electron donor in these experiments was 20 mM formate, and the initial concentration of nitrate was 1 mM. At the end of each reaction, nitrate was again added to confirm that the initial rate of nitrate reduction could be re-established and, therefore, that the enzyme had not been inactivated during the incubation period.

## RESULTS

### General strategy

A series of *E. coli* double mutants were constructed to compare the physiological roles of Nap and the membrane-associated nitrate reductases, nitrate reductase A and nitrate reductase Z (Table 1). First we assessed the ability of strains expressing only one of the three nitrate reductases to grow anaerobically in the presence of nitrate and the non-fermentable carbon source, glycerol. Next we investigated whether a strain that expresses only Nap can grow continuously in the presence of excess nitrate, conditions known to mediate NarL-dependent repression of *nap* gene expression [14]. Competition experiments were then designed to define the growth conditions that give one strain a selective advantage over the other. Finally, the abilities of the double mutants to reduce very low concentrations of nitrate were compared.

### Nitrate-dependent anaerobic growth in batch culture of *E. coli* strains expressing only a single nitrate reductase

*E. coli* strains JCB4011, 4021 and 4022 express only nitrate reductase A, Nap or nitrate reductase Z respectively. All are derived from the parental strain, RK4353. Each of the double mutants, the parental strain and a triple mutant, strain JCB4023, were grown anaerobically in GM medium supplemented with glycerol as a non-fermentable carbon source and 20 mM nitrate as the terminal electron acceptor. Both the strain expressing nitrate reductase A and the parental strain grew rapidly, reaching the stationary phase of growth after approx. 8 h (Figure 1). In contrast, no significant growth of the nitrate reductase Z strain or the triple mutant was detected. Growth of the strain expressing only Nap was also very limited.

### Effect of a *narL* mutation on nitrate-dependent anaerobic growth on a non-fermentable carbon source

If the poor growth of the Nap$^+$ strain in batch culture is due solely to nitrate-mediated repression of *nap* operon expression by NarL, a *narL nap*$^+$ mutant strain should be able to grow better

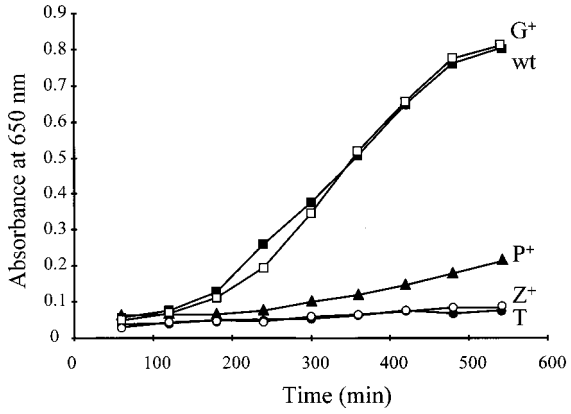


**Figure 1 Growth of double mutants expressing either nitrate reductase A (G$^+$), nitrate reductase Z (Z$^+$) or Nap (P$^+$) compared with the parental strain expressing all three nitrate reductases (wt) and with a triple mutant (T) devoid of nitrate reductase activity**

Strains were grown anaerobically in a defined, minimal medium with glycerol as the non-fermentable carbon source and nitrate as the terminal electron acceptor. Data are typical of those obtained from at least five independent experiments.

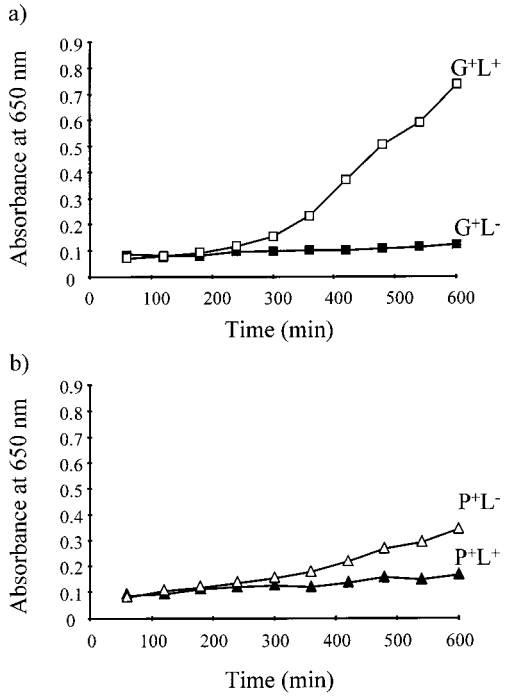


**Figure 2 Effect of a *narL* mutation on anaerobic growth of strains expressing either nitrate reductase A alone, or Nap alone, in the presence of glycerol and nitrate**

Other details are as for Figure 1.

in nitrate-supplemented broth cultures than the isogenic *narL*$^+$ *nap*$^+$ strain. To test this prediction, a *narL* mutation was introduced into the *narL*$^+$ *nap*$^+$ strain, JCB4021, to generate strain JCB4024; and into the corresponding *narL*$^+$ *narG*$^+$ strain, JCB4011, to generate strain JCB4012. The ability of both strains to grow in batch culture with glycerol as the non-fermentable carbon source was then compared with that of the corresponding *narL*$^+$ strain. As expected, the *narL* mutation severely decreased the ability of the strain expressing nitrate reductase A to grow by nitrate respiration; conversely, the *narL nap*$^+$ strain grew better than the isogenic *narL*$^+$ *nap*$^+$ strain (Figure 2). These results confirm that the failure of the *narL*$^+$ *nap*$^+$ strain to grow in batch culture with glycerol and nitrate was partly due to the NarL-dependent repression of *nap* operon expression by nitrate [12,13].

### Competition in a chemostat between strains expressing either nitrate reductase A or the periplasmic nitrate reductase

During continuous growth in a chemostat, the growth rate of bacteria is the dilution rate, which is defined by the rate at which fresh growth medium is supplied. The yield is usually determined by the concentration of the growth-limiting nutrient in the feed. For any culture during steady-state growth, the Monod equation [21] relates the growth rate to the maximum possible growth rate, and to the affinity constant ($K_s$) and the residual concentration of the growth-limiting substrate. In a mixed culture of two or more strains, the strain with the highest growth rate under the conditions imposed will have a selective advantage.

A chemostat was inoculated with approximately equal concentrations of strains JCB4011 (expressing nitrate reductase A) and JCB4021, which expresses only Nap. This mixed culture was grown at a dilution rate of $0.06 \pm 0.02$ h$^{-1}$ with excess glycerol, a limiting concentration of nitrate and fumarate as an auxiliary terminal electron acceptor. When a steady state had been achieved, more than 90% of the bacteria in the culture were erythromycin-resistant and therefore expressed the periplasmic nitrate reductase. The concentration of nitrate in the feed medium was then increased from 5 mM to 30 mM and the glycerol concentration decreased from 0.4% (v/v) to a growth-limiting concentration of 0.1% (v/v). Within two mean generation times, the proportion of erythromycin-sensitive bacteria expressing nitrate reductase A had increased from less than 10 to more than 50%. During steady-state growth, $98 \pm 2$% of the bacteria were erythromycin-sensitive and therefore expressing nitrate reductase A.

These trends were entirely reproducible in independent chemostat experiments. Furthermore, in the reverse experiment, the strain expressing nitrate reductase A dominated the culture during the early stages of the experiment when the feed medium contained 30 mM nitrate, but was largely replaced by the Nap$^+$ strain during growth with limiting nitrate. However, during prolonged growth of the mixture of erythromycin-resistant, Nap$^+$ and erythromycin-sensitive bacteria expressing only nitrate reductase A with limiting nitrate, the proportion of erythromycin-sensitive bacteria increased unpredictably. This suggested that the erythromycin-resistance cassette was being lost spontaneously from the Nap$^+$ strain to generate revertants that could additionally express nitrate reductase A. To investigate this possibility, strain JCB4021 was inoculated alone into a nitrate-limited chemostat supplied with excess glycerol, 5 mM nitrate and 40 mM fumarate. Only erythromycin-resistant bacteria were detected after five generations of growth and the nitrate reductase activity was below the limits of detection with Methyl Viologen as the electron donor. However, as soon as the concentration of nitrate in the feed was increased to 30 mM and glycerol was the growth-limiting nutrient, erythromycin-sensitive bacteria were isolated from the culture and rates of nitrate reduction with Methyl Viologen as the electron donor increased to those typical of nitrate reductase A. The results from this control experiment revealed that, although spontaneous reversion to *narG*$^+$ was occurring during nitrate-limited growth, bacteria able to express nitrate reductase A were at a selective advantage only during growth with a high concentration of nitrate in the feed. This strongly suggested that, during nitrate-limited growth, strains which express the periplasmic nitrate reductase have a selective advantage relative to those that express nitrate reductase A.

### Selective advantage during nitrate-limited growth of a strain expressing the periplasmic nitrate reductase

To avoid the problem of reversion due to loss of the erythromycin-resistance cassette, we constructed strain JCB4031 from which most of the *narGHJI* operon had been deleted. This strain also carries the *narZ*::Ω insertion and therefore expresses only Nap. In the experiments described above, during growth with limited glycerol nitrate was not available in excess because all of the nitrate had been converted into nitrite and some of the nitrite had been reduced further to ammonia. In a preliminary experiment, we therefore confirmed that strain JCB4031 expressing only a periplasmic nitrate reductase can grow in the presence of excess nitrate and in the absence of fumarate. This was achieved using 50 mM nitrate and 0.1% (v/v) glycerol.

Strains JCB4011 (expressing nitrate reductase A) and 4031 (Nap$^+$) were inoculated to approximately equal densities into the fermenter and supplied with excess glycerol and limiting nitrate as the only terminal electron acceptor. After 49 h at a dilution rate of 0.05 (almost four mean generation times), the medium

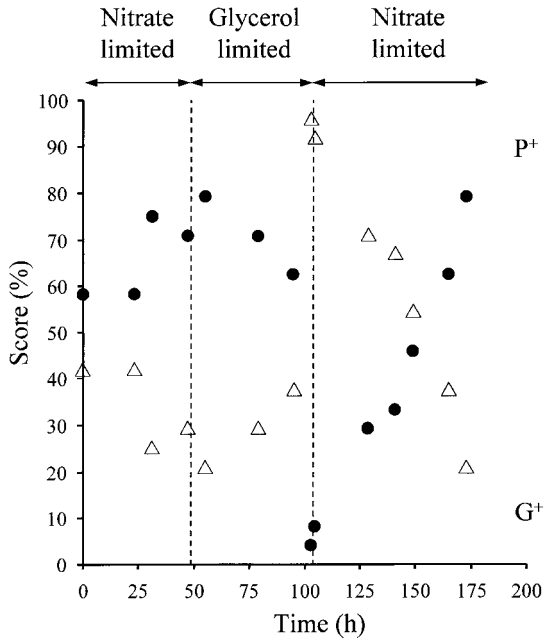


**Figure 3 Competition between double mutants expressing either nitrate reductase A alone (strain JCB4011), or Nap alone (strain JCB4031), in a chemostat with a nitrate-limited or a nitrate-sufficient feed medium**

The non-fermentable carbon source was supplied in excess when nitrate was the growth-limiting nutrient and was growth-limiting during nitrate-sufficient growth (50 mM nitrate in the feed). The dilution rate was 0.05; the pH was $6.8 \pm 0.1$. ●, percentage of the population that were $Nap^+$; △, percentage of the population that were expressing nitrate reductase A. Note the extreme points after 101 and 103 h of continuous growth when less than 10% of the population were the $Nap^+$ strain, JCB4031. Each point represents the percentage of the strain in a sample of 24 colonies assayed at random. Replicate assays by independent workers gave identical results within variations expected on the basis of a random sample.

was changed so that glycerol was the growth-limiting nutrient and nitrate was available in excess. After a further four mean generation times, the nitrate-limiting medium was again supplied for a further 73 h (five mean generation times). Throughout the experiment, the following were monitored: the pH of the culture, the purity and bacterial density, the ratio of bacteria expressing either Nap or nitrate reductase A, and rates of nitrate reduction by formate, glycerol and Methyl Viologen.

The pH of the culture remained almost constant at $6.8 \pm 0.1$. The biomass concentration in the initial, nitrate-limited culture was $0.35 \pm 0.03$ g·l$^{-1}$ ($D_{650}$ of 0.8–0.9), but subsequently decreased to $0.2 \pm 0.02$ g·l$^{-1}$ ($D_{650}$ of 0.45–0.55) when excess nitrate was supplied. Neither nitrate nor nitrite could be detected in the culture during nitrate-limited growth, but $20 \pm 3$ mM nitrite and $10 \pm 2$ mM nitrate were detected in the glycerol-limited culture, confirming that nitrate was available in excess.

The initial ratio of bacteria expressing either Nap or nitrate reductase A was 14:10 (24 colonies screened individually; Figure 3). This ratio increased to 4:1 during nitrate-limited growth, but then declined rapidly during nitrate-sufficient growth until, after a further 54 h, the $Nap^+$ strain had decreased to less than 10% of the population. (Note the extreme values for the sample taken after 101 h of growth, which were checked using a replicate sample taken 2 h later.) The Methyl Viologen-dependent nitrate reductase activity increased four-fold during nitrate-sufficient growth due to induction of nitrate reductase A activity in strain JCB3011 and repression of the already low activity in the residual population of the $Nap^+$ strain, JCB4031 (Table 4). During the final, nitrate-limited, phase of the experiment, the ratio of bacteria expressing either Nap or nitrate reductase A again increased, confirming the selective advantage of bacteria able to express a periplasmic nitrate reductase under these conditions (Figure 3). This was again accompanied by a decrease in the Methyl Viologen-dependent nitrate reductase activity (Table 4).

High rates of formate- or glycerol-dependent nitrate reduction were detected with bacteria from both the nitrate-sufficient and the nitrate-limited culture. However, the glycerol-dependent rate more than doubled during nitrate-limited growth (Table 4). The trends were fully confirmed by other data not included in the Table. Rates of nitrate reduction measured with the nitrate electrode did not correlate with the corresponding Methyl Viologen-dependent rate of nitrate reduction, reflecting the fact that Methyl Viologen is a poor electron donor to NapA. As expected, the Methyl Viologen-dependent rates of nitrate reduction of bacteria from the nitrate-limited culture were almost too low to be measured accurately, consistent with this culture being dominated by the strain able to express only Nap (Table 4 and Figure 3).

### Saturation constant for nitrate of strains expressing either the periplasmic nitrate reductase or nitrate reductase A

Strains JCB4011 and 4031, expressing either nitrate reductase A or Nap, were grown as batch cultures to the middle of the

**Table 4 Yields of bacteria, concentrations of nitrate and nitrite in the fermenter, and rates of nitrate reduction by bacteria from a chemostat during nitrate-limited or nitrate-sufficient growth**

Rates of nitrate reduction by Methyl Viologen are nmol of nitrite formed · min$^{-1}$ · mg of bacterial dry weight$^{-1}$. Rates of nitrate reduction by formate and glycerol are nmol of nitrate reduced · min$^{-1}$ · mg of bacterial dry weight$^{-1}$.

| | | | | | Rate of nitrate reduction by: | | |
|---|---|---|---|---|---|---|---|
| Sample h* | Limiting nutrient | $D_{650}$ | [$NO_3^-$] (mM) | [$NO_2^-$] (mM) | Methyl Viologen | Formate | Glycerol |
| 0 | Nitrate | 0.865 | < 0.1 | < 0.1 | 25 | nd† | nd |
| 79 | Glycerol | 0.647 | 9.4 | 19.7 | 64 | 106 | 67 |
| 101 | Glycerol | 0.604 | nd | nd | 97 | 136 | 64 |
| 175 | Nitrate | 0.500 | < 0.1 | < 0.1 | 17 | 143 | 141 |

* Number of hours after the chemostat pump was started before the sample was taken.
† nd, not determined.

exponential phase in the presence of glycerol, fumarate and nitrate, harvested, and assayed for formate-dependent nitrate reduction using the nitrate electrode. Data from the progress curves from these reactions were fitted to the integrated form of the Monod equation and the saturation constants ($K_s$) were calculated as described by Wharton and Eisenthal [20]. The estimated $K_s$ was 15 $\mu$M ($\pm 2$ $\mu$M; eight determinations) for the strain expressing only the periplasmic nitrate reductase, and 50 $\mu$M ($\pm 6$ $\mu$M; ten determinations) for the strain expressing nitrate reductase A.

## DISCUSSION

Over 40 years of research have led to the detailed characterization of the anaerobically induced, membrane-associated nitrate reductase encoded by the *narGHJI* operon of *E. coli*. Although its role in energy conservation during anaerobic growth in the presence of nitrate is well established, no-one has previously investigated its role in a strain that lacks both nitrate reductase Z and Nap. After anaerobic growth in batch cultures with high concentrations of nitrate, mutants defective in expression of nitrate reductase A reduce nitrate at a rate of less than 10 % of that of the parental strain. This has led to the description of nitrate reductase A as the major nitrate reductase of *E. coli* [22]. Data reported in Figure 1 fully support the role of nitrate reductase A in providing the major source of energy for anaerobic growth in batch culture with a non-fermentable carbon source and nitrate as the terminal electron acceptor. They confirm the inability of the two alternative nitrate reductases to substitute for nitrate reductase A under the conditions used for these experiments. Furthermore, the inability of Nap to substitute for nitrate reductase A is clearly due to NarL-mediated inhibition of *nap* operon transcription during growth in the presence of nitrate (Figures 1 and 2).

Batch cultures supplemented with 20 mM, 50 mM or even 100 mM nitrate have been used for most of the published work on the role of nitrate reductase A in enteric bacteria. However, the concentrations of nitrate at various sites in the natural environment for enteric bacteria, the human body, rarely exceed the micromolar range [23]. These conditions are not achievable in laboratory batch cultures, but require continuous-culture techniques such as those used in the present work. We have estimated the $K_s$ values for nitrate to be about 50 $\mu$M for nitrate reductase A and about 15 $\mu$M for Nap, and the maximum specific growth rates for nitrate-limited cultures expressing either nitrate reductase A or Nap are about 0.5 $h^{-1}$. At a dilution rate of 0.05 in a nitrate-limited chemostat, the steady-state concentration of nitrate for the strain expressing only Nap, calculated from the Monod equation [21], is 1.67 $\mu$M, far closer to that encountered by the bacteria in their natural environment [23]. Under these conditions, due to its higher $K_s$ for nitrate, the growth rate of the strain expressing only nitrate reductase A would be 0.016. As this is only one-third of the imposed growth rate, this fully explains why this strain was unable to compete against the $Nap^+$ strain and was gradually washed out of the chemostat during nitrate-limited growth. We conclude that both the kinetic characteristics and the transcription control enable Nap to be the effective enzyme in scavenging low concentrations of nitrate, whereas nitrate reductase A favours more rapid and more efficient growth when nitrate is available in excess.

Further examples of bacteria with genes for periplasmic nitrate reductases are rapidly becoming recognized from results of current genome sequencing projects. Several patterns are beginning to emerge. First, periplasmic nitrate reductases have been found in most bacteria reported to denitrify during aerobic growth; consequently they are the major enzymes catalysing the first step in aerobic denitrification [24,25]. The best-characterized Nap systems, those of *Thiosphaera pantotropha* (also designated *Paracoccus denitrificans* GB17 and recently renamed *Paracoccus pantotrophus*) and *Ralstonia* (formerly *Alcaligenes*) *eutropha* are examples of this group. A further role for Nap in *T. pantotropha* appears to be to maintain redox balance during aerobic growth on a highly reduced carbon source such as butanol [26]. This is similar to the redox balancing role proposed for Nap in the photosynthetic bacteria *Rhodobacter capsulatus* [27] and *Rhodobacter sphaeroides* DSM 158 [28]. At the other extreme are the Nap systems of enteric bacteria such as *E. coli* [7,14] which are expressed only during anaerobic growth and initiate not denitrification, but the dissimilatory reduction of nitrate to ammonia.

There is also a correlation at the genetic level between *nap* operon structure and the physiological roles of the encoded enzymes. The *nap* operons of the aerobic denitrifiers sequenced to date are relatively simple, consisting of the five genes *napEDABC* [5,6]. In contrast, *nap* operons from bacteria that express Nap activity only during anaerobic growth are more complex, those of *Rhodobacter sphaeroides* DSM 158 and *E. coli* each consisting of seven genes, *napKEFDABC* and *napFDAGHBC*, respectively [7,8]. The probable occurrence of further groups is indicated by the properties of the anaerobically induced nitrate reductase of a rhizobium strain that was originally designated *Pseudomonas* strain G-179 [10]. This strain apparently synthesizes only a periplasmic nitrate reductase that is essential for anaerobic denitrification. Note that the structure of the *nap* operon from this bacterium, *napEFDABC*, differs from those of other groups mentioned above.

Results from genome sequencing projects reveal that only the *nap* genes have been conserved in several other pathogens such as *Haemophilus influenzae*, *Vibrio cholerae*, *Yersinia pestis* and *Campylobacter jejuni*. This suggests that Nap might play an important role in pathogenic bacteria. These data reinforce another correlation, the domination of nitrate-rich, carbon-deficient environments such as soil and water by denitrifying bacteria. In contrast, bacteria that reduce nitrate via nitrite to ammonia dominate carbon-rich, nitrate-deficient environments such as polluted estuaries and the human body [29–33]. Furthermore, the co-ordinated expression of Nap with a periplasmic nitrite reductase that catalyses the reduction of nitrite to ammonia ensures that the maximum number of electrons are consumed for each molecule of nitrate reduced. This again is in contrast with denitrification.

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