Role of the *Escherichia coli* nitrate transport protein, NarU, in survival during severe nutrient starvation and slow growth

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Escherichia coli K-12 strains expressing either NarU or NarK as the only nitrate transport protein are both able to support nitrate-dependent anaerobic growth. The *narK* gene is highly expressed during anaerobic growth in the presence of nitrate, consistent with a role for NarK in nitrate transport coupled to nitrate reduction by the most active nitrate reductase encoded by the adjacent *narGHJI* operon. The physiological role of NarU is unknown. Reverse transcriptase PCR experiments established that, unlike the monocistronic *narK* gene, *narU* is co-transcribed with *narZ* as the first gene of a five-gene *narUZYWV* operon. The *narK* and *narU* genes were fused in-frame to a *myc* tag: the encoded fusion proteins complemented the nitrate-dependent growth defect of chromosomal *narK* and *narU* mutations. A commercial anti-Myc antibody was used to detect NarK and NarU in membrane fractions. During anaerobic growth in the presence of nitrate, the quantity of NarU-Myc accumulated during exponential growth was far less than that of NarK-Myc, but NarU was more abundant than NarK in stationary-phase cultures in the absence of nitrate. Although the concentration of NarU-Myc increased considerably during the post-exponential phase of growth, NarK-Myc was still more abundant than NarU-Myc in stationary-phase bacteria in the presence of nitrate. In chemostat competition experiments, a strain expressing only *narU* had a selective advantage relative to a strain expressing only *narK* during nutrient starvation or very slow growth, but *NarK*⁺ bacteria had a much greater selective advantage during rapid growth. The data suggest that NarU confers a selective advantage during severe nutrient starvation or slow growth, conditions similar to those encountered *in vivo*.

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INTRODUCTION

Under different growth conditions, *Escherichia coli* expresses three different nitrate reductase activities. The periplasmic nitrate reductase is expressed primarily during anaerobic growth in the presence of very low concentrations of nitrate (Choe & Reznikoff, 1993; Rabin & Stewart, 1993; Grove *et al.*, 1996; Potter & Cole, 1999; Wang *et al.*, 1999). The active sites of the other two nitrate reductases are located in the cytoplasm (Showe & DeMoss, 1968; MacGregor & Christopher, 1978; Graham *et al.*, 1981), so nitrate must be transported across the cytoplasmic membrane to be reduced to nitrite by these enzymes. Nitrate reductase A, encoded by the *narGHJI* operon, is repressed during aerobic growth, but it is strongly induced during anaerobic growth in the presence of high concentrations of nitrate (Stewart, 1988; Berks *et al.*, 1995; Potter *et al.*, 1999; Wang *et al.*, 1999). Under these growth conditions, nitrate reductase A is the most active of the three nitrate reductases. The third nitrate

reductase, encoded by the *narZYWV* operon, is structurally very similar to nitrate reductase A (Blasco *et al.*, 1990; Bonnefoy *et al.*, 1997), but is expressed extremely weakly during both aerobic and anaerobic growth (Iobbi *et al.*, 1987). Although expression of nitrate reductase Z is induced during entry into the stationary phase of growth, its activity is still at the lower limits of detection by most biochemical assays (Iobbi-Nivol *et al.*, 1990; Bonnefoy *et al.*, 1997; Chang *et al.*, 1999; Potter *et al.*, 1999). Consequently, nitrate reductase Z contributes very little to the overall rate of nitrate reduction by *E. coli*, and is sufficient to support only a very low rate of nitrate-dependent anaerobic growth with glycerol as the non-fermentable carbon source (Potter *et al.*, 1999).

There are two nitrate transport proteins in *E. coli*, NarK and NarU (Clegg *et al.*, 2002). Both NarK and NarU, as well as a third protein, NirC, were all shown to catalyse nitrite transport, but the different physiological roles of these three proteins in nitrite transport were not defined. Nitrite accumulates in the medium during anaerobic growth of *narK*⁺ strains in the presence of nitrate, irrespective of whether *narU* or *nirC* are expressed (Noji *et al.*, 1989; DeMoss & Hsu,

Abbreviation: FNR, regulator of fumarate and nitrate reduction.

Two supplementary tables are available with the online version of this paper.

1991; Rowe *et al.*, 1994; Clegg *et al.*, 2002). This is consistent with a nitrate:nitrite antiport role for NarK, but does not exclude other transport mechanisms, for example, nitrate:H⁺ symport, or nitrite:H⁺ export. Although less nitrite accumulates during anaerobic exponential growth of a *narK* mutant than the parental strain in the presence of nitrate, NarU expressed from a multicopy plasmid fully complements the complex phenotypes of the *narK* mutant, including nitrite accumulation during exponential growth (Bonnefoy *et al.*, 1997; Clegg *et al.*, 2002). This suggests that NarU duplicates the functions of NarK, except that it is expressed at a much lower level that limits the rate of nitrate uptake and reduction by a *narK* mutant.

In bacteria, there are often strong correlations between the rate of transcription and the quantity of protein synthesized, and between how transcription is regulated and the physiological roles of the encoded proteins. The *narK* and *narU* genes are both located immediately upstream from four structural genes encoding the alternative nitrate reductases, nitrate reductase A and nitrate reductase Z, respectively. It is well established that *narK* and *narGHJI* are organized as two operons transcribed in the same orientation, but with 500 bp of intergenic DNA that includes a rho-independent *narK* transcription terminator, and binding sites for regulation by the oxygen-responsive transcription factor, FNR, and the nitrate–nitrite-responsive two-component regulatory system, NarX–NarL (Stewart & Parales, 1988; Kolesnikow *et al.*, 1992). In contrast, *narU* and *narZ* are separated by only 81 bp of DNA that lacks recognizable transcription termination or promoter sequences. However, a potential RpoS-dependent promoter is located 116 bp upstream of the *narU* transcription start site. This strongly suggests that *narU* might be the first gene of a five-gene *narUZYWV* operon, and therefore that NarU might be important for anaerobic survival under conditions in which the RpoS regulon is activated. We now report results of reverse transcriptase PCR experiments to determine whether *narU* is transcribed as a polycistronic message with the RpoS-regulated *narZ* operon, and whether the relative abundance of NarU and NarK proteins

correlates with the previously reported nitrate reductase activities of strains that synthesize only nitrate reductase Z or nitrate reductase A (Potter *et al.*, 1999). Chemostat competition experiments were designed to identify conditions in which a strain expressing only *narU* has a selective advantage over a strain expressing only *narK*, especially during severe nutrient limitation under conditions in which the RpoS regulon is activated.

METHODS

Bacterial strains, plasmids and oligonucleotides. *Escherichia coli* K-12 strain JCB4011 is a derivative of strain RK4353 that expresses nitrate reductase A, encoded by the *narGHJI* operon, but neither the periplasmic nitrate reductase nor nitrate reductase Z (Potter *et al.*, 1999: Table 1). As the catalytic site of NarG is located on the cytoplasmic side of the membrane, nitrate reduction by this strain and its derivatives is totally dependent on active nitrate transport by NarK or NarU. Strains JCB4014, 4016 and 4018 are derivatives of JCB4011 with deletions in *narK*, *narU*, or both *narU* and *narK*, respectively (Clegg *et al.*, 2002). Note that the fusaric acid method was used to cure the tetracycline resistance determinant from Tn10 in strain JCB4014, which is therefore a stable *narK* deletion mutant. This strain was checked by PCR for loss of the *narK*⁺ gene, and by biochemical assays for retention of the adjacent *narXL* and *narG* genes required for induction of nitrate reductase A activity during anaerobic growth in the presence of nitrate (see Clegg *et al.*, 2002, for further details). Strain JCB4016 carries a deletion–insertion mutation in *narU*: it was constructed in strain RK4353 using the method of Datsenko & Wanner (2000), and transferred into strain JCB4011 by bacteriophage P1-mediated transduction. Kanamycin-resistant transductants were checked by PCR for the deletion–insertion mutation. Strain JCB4520 is a Δ *nirC* Δ *nrfAB* derivative of JCB4018 and therefore lacks all three nitrate and nitrite transport proteins, NarK, NarU and NirC, as well as the periplasmic nitrite reductase (Table 1).

E. coli strain RV and its *pcnB* derivative, JCB301, in which the plasmid copy number is decreased to about one (Table 1), were used for Western blotting experiments.

Oligonucleotide primers were obtained from Alta Bioscience, University of Birmingham, UK: their sequences are listed in Supplementary Table S1, available with the online version of this paper. Plasmid pSJC901 was constructed by cloning the PCR-generated

Table 1. *E. coli* K-12 strains used in this study, their genotype and source

Strain	Genotype	Source
JCB4011	RK4353 Δ <i>napAB</i> Δ <i>narZ</i> :: Ω	Potter <i>et al.</i> (1999)
JCB4014	As JCB4011, but also Δ <i>narK</i>	Clegg <i>et al.</i> (2002)
JCB4016	As JCB4011, but also Δ <i>narU</i> :: <i>kan</i>	Clegg <i>et al.</i> (2002)
JCB4018	JCB4011 Δ <i>narK</i> Δ <i>narU</i> :: <i>kan</i>	Clegg <i>et al.</i> (2002)
JCB4514	As JCB4014 but also Δ <i>nirC</i>	Clegg <i>et al.</i> (2002)
JCB4516	As JCB4016, but also Δ <i>nirC</i>	Clegg <i>et al.</i> (2002)
JCB4518	As JCB4018, but also Δ <i>nirC</i>	Clegg <i>et al.</i> (2002)
JCB4520	As JCB4518, but also Δ <i>nrfAB</i>	This study
RK4353	Δ <i>lacU169</i> <i>araD139</i> <i>rpsL</i> <i>gyrA</i> <i>non</i>	Stewart (1982)
RV	Prototrophic F [−] but Δ <i>lacZYA</i>	Laboratory stocks
JCB301	<i>pcnB</i> derivative of RV	Clegg <i>et al.</i> (2002)

coding sequence of *narU* and approximately 1 kb of upstream DNA into the *Pst*I site of pGEM T Easy (Clegg *et al.*, 2002). In pJCB901, the translation stop codon of *narU* has been replaced by a synthetic in-frame Myc tag using the primer NarU Myc2. Similarly, plasmids pWJ902 and pJCB902, encoding NarK and NarK-Myc, respectively, were constructed using primers NarK up271 and either NarK down39 or NarK myc.

Growth conditions and preparation of subcellular fractions.

Inocula were grown with aeration in 20 ml Luria broth (LB; Sambrook *et al.*, 1989) in 100 ml conical flasks. After 4 h growth at 37 °C, these cultures were transferred into 900 ml minimal medium in a 1 l conical flask, or 1.9 l medium in a 2 l conical flask, or for continuous culture experiments into 100 ml of the specified medium in an aspirator bottle. Cultures were incubated at 37 °C without aeration either until the mid-exponential phase of growth (typically to an OD₆₅₀ of 0.6, which corresponds to 0.24 mg bacterial dry mass ml⁻¹), or into stationary phase, where noted. Optical density readings were measured using 1 cm light path cuvettes and a Camspec M501 spectrophotometer. The defined medium was the minimal salts medium described by Pope & Cole (1984). Media were supplemented where appropriate with the following antibiotics: ampicillin, 100 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; chloramphenicol, 34 µg ml⁻¹; or tetracycline, 15 µg ml⁻¹. Media for anaerobic cultures were supplemented with either glucose or glycerol to a final concentration of 0.4% (v/v) (unless stated otherwise) and with 5 or 20 mM nitrate, as noted.

Bacteria from 2 l batch cultures were harvested by centrifugation at 9000 g for 1 min, resuspended in 15 ml 50 mM Tris/HCl pH 8.0 containing a mixture of protease inhibitors (100 µM PMSF, 1 µM pepstatin, 1 µM leupeptin and 1 µM EGTA) and periplasmic proteins were released by sucrose/lysozyme/EDTA treatment, as described previously (McEwan *et al.*, 1984). The spheroplast pellet was resuspended in 6–15 ml (the volume depending on the density of the culture at harvest) 50 mM Tris/HCl pH 8.0 containing the protease inhibitor mixture, broken by two passages at approximately 50 MPa through a French pressure cell and centrifuged for 10 min at 8000 g. The supernatant was collected for a further centrifugation at 120 000 g for 30 min. The translucent pellet of cytoplasmic membranes was resuspended in approximately 400 µl 1 mM Tris/HCl pH 8.0, and the protein concentration was determined using the Folin method.

Continuous culture competition experiments. To establish mixed cultures of strains JCB4014 (*narU*⁺) and JCB4016 (*narK*⁺) for competition experiments, each strain was grown separately without aeration in 100 ml of the required medium in aspirator bottles that were incubated for 4–6 h at 37 °C. These cultures were then transferred into a 2 l vessel of an MBR Mini-Bioreactor fermenter containing 800 ml of the same medium. The feed pump was started before the bacteria had entered the stationary phase, and bacteria were grown continuously at the required dilution rate, usually in the range 0.02–0.1 h⁻¹. At intervals, samples taken aseptically were checked for purity by plating for single colonies onto nutrient agar (NA), or microscopically following staining by the Gram method. Serial dilutions were also plated onto unsupplemented nutrient agar to obtain 100–400 individual colonies. After overnight growth, 36 colonies were transferred to square grids on unsupplemented NA plates, and after 8–16 h at 37 °C, were replica plated onto NA alone and NA supplemented with kanamycin. The *narU*⁺ strain, JCB4014, is kanamycin sensitive, but strain JCB4016 (*narK*⁺) is kanamycin-resistant, so the ratio of the two strains during each stage of growth could be calculated. Control experiments established that the kanamycin resistance cassette did not affect growth rates during aerobic growth, during anaerobic fermentative growth with glucose as the carbon source, or during anaerobic growth in media supplemented with glycerol and fumarate as the carbon and energy source.

Reverse transcriptase PCR. Anaerobic cultures of strain RK4353 were grown to early stationary phase (OD₆₅₀ about 0.9) in Luria broth supplemented with 0.4% (v/v) glycerol and 20 mM nitrate. Total RNA was isolated using an RNase spin cell mini-kit (Biolone) following the manufacturer's instructions, except that bacteria were incubated with lysozyme for 10 min. The RNA was dissolved in 50 µl distilled water and stored at -80 °C. The SUPERScript First-Strand Synthesis System (Invitrogen) was used to synthesize first-strand DNA from 5 µl total RNA, 5 µl 50 ng µl⁻¹ random hexamer primers and 1 µl dNTP mix in a diethyl pyrocarbonate (DEPC)-treated Eppendorf tube incubated at 65 °C for 5 min. After 1 min on ice, 2 µl reverse transcriptase buffer, 4 µl 25 mM MgCl₂, 2 µl dithiothreitol and 1 µl RNase OUT were added. After a further 2 min at room temperature, 1 µl SUPERScript II reverse transcriptase was added. After 10 min at room temperature, 50 min at 42 °C and 15 min at 70 °C, 1 µl RNase H was added, and samples were incubated at 37 °C for 20 min. The cDNA was amplified by PCR using BioTaq DNA polymerase.

Rates of nitrate reduction by washed bacterial suspensions.

Harvested bacteria were resuspended in 50 mM phosphate buffer, pH 7.3, and assayed for nitrate reduction by formate using a nitrate electrode (Potter *et al.*, 1999; Clegg *et al.*, 2002). The data quoted in the text were the means and standard deviations for up to five independent biological replicates, each assayed in duplicate.

SDS-PAGE and Western blotting. Membrane proteins that had been resolved by SDS-PAGE (Grove *et al.*, 1996) were transferred electrophoretically using a Bio-Rad Trans-D semi-dry blotter onto a PVDF membrane (Millipore) at 10 V for 1.5 h in a transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20%, v/v, methanol). The membrane was removed and gently agitated overnight at 4 °C in 100 ml blocking solution (50 mM Tris/HCl pH 7.5, 150 mM NaCl, plus 5%, w/v, non-fat dry milk). The blocked membrane was washed three times for 5 min with gentle agitation in 80 ml 50 mM Tris/HCl pH 7.5, 150 mM NaCl, plus 0.1% Tween 20 (TBST), transferred to a plastic bag containing mouse anti-Myc antibody diluted 1:5000 in 20 ml TBST buffer plus 5% non-fat dry milk, and incubated with gentle agitation for 2 h at room temperature. After a further three washes for 5 min in TBST with gentle agitation, the membrane was incubated with horseradish-peroxidase-labelled anti-mouse IgG secondary antibody (Invitrogen Life Technologies), diluted 1:5000 in 20 ml TBST buffer plus 5% non-fat dry milk, and incubated with gentle agitation for 1 h at room temperature. After a further three washes in 80 ml TBST, immunoconjugates were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences RPN2133).

RESULTS

Detection of transcription across the *narU*–*narZ* intergenic region

RT-PCR was used to determine whether *narU* transcription proceeds across the intergenic region into the *narZWYV* operon or, like *narK*, whether it is expressed as a separate monocistronic operon. The DNA sequences of the *E. coli* *narK* and *narU* genes are 75% identical, and *narG* and *narZ* are 72% identical. Six primers specific for either *narK* or *narU* and two reverse primers specific for either *narZ* or *narG* were designed and checked for target specificity (Fig. 1a). In preliminary experiments with chromosomal DNA from *E. coli* RK4353 as template, it was confirmed that the appropriate primer pairs could be used to amplify the expected *narK* or *narK*–*narG* fragments, but these primers

did not amplify the corresponding *narU*–*narZ* fragments. Similarly, the *narU* and *narZ* primer pairs were shown to be specific for amplification of the corresponding *narU* or *narU*–*narZ* DNA fragments.

Having established that the primers were suitable for distinguishing between transcripts from the *narK* and *narU* operons, strain RK4353 was grown anaerobically to early stationary phase in LB supplemented with 0.4 % glucose. RNA was isolated and used as template for cDNA synthesis by reverse transcriptase-PCR. The final PCR products were separated and visualized on a 0.8 % agarose gel (Fig. 1b). No PCR product was obtained in the absence of added RNA (lane 3), or with the primer pair that would detect a transcript (or contaminating DNA) across the *narK*–*narG* intergenic region (lane 5). Neither was a product obtained in controls without reverse transcriptase, confirming that the RNA was not contaminated with chromosomal DNA (data not shown). Positive controls using primer pairs specific for *narK* or *narU* gave readily detectable cDNA products of the expected size (Fig. 1b, lanes 6 and 7). A PCR product corresponding to cDNA amplified from the intergenic region spanning *narU* and *narZ* was also obtained (lane 4), indicating that *narU* transcription proceeds across this intergenic boundary. We conclude that, unlike the monocistronic *narK*, *narU* is by definition the first gene of a five-gene *narUZYWV* operon, as proposed by Bonnefoy & DeMoss (1994). Furthermore, primer extension

experiments with two different primers complementary to the 5' end of *narZ* failed to reveal a second transcript initiating within the short intergenic region between *narU* and *narZ*.

Relative quantities of NarK and NarU under different growth conditions

Previous studies had indicated that the rate of nitrate-dependent growth of an *E. coli narK* mutant is limited by the rate of nitrate uptake by NarU (DeMoss & Hsu, 1991; Clegg *et al.*, 2002). Having established that *narU* is the first gene of the RpoS-dependent *narZ* operon, it was likely that little NarU would be synthesized during exponential growth, but its abundance would increase during the stationary phase. We therefore attempted to determine the relative quantities of NarK and NarU in bacteria harvested during the exponential and stationary phases of growth.

Although *narK* is optimally expressed during anaerobic growth in the presence of excess nitrate (Stewart & Parales, 1988; Kolesnikow *et al.*, 1992), analysis of either whole-cell or membrane proteins by SDS-PAGE failed to reveal a protein band that could reliably be identified as NarK. Western analysis was therefore used to develop a method to detect both NarK and NarU and to provide an indication of the relative quantities of the two proteins in different strains and after growth under different conditions. In all

Fig. 1. (a) Location of primers used to detect *narK* and *narU* transcripts. Primers NarU FORWARD and NarU Cterm are forward primers that recognize the 5' or 3' ends of *narU*, respectively. Primer NarU REVERSE and NarZ Nterm are reverse primers that bind within *narU* and *narZ*. Similarly, primers NarK FORWARD and NarK Cterm recognize the 5' and 3' ends of the *narK* coding strand; NarK REVERSE and NarG Nterm are reverse primers that bind within *narK* and *narG*. (b) RT-PCR products demonstrating transcription across the *narU*–*narZ* intergenic region. Lanes: 1, 1 kb ladder; 2, 100 bp ladder; 3, negative control with no RNA added during reverse transcription; 4, amplification of the *narU*–*narZ* intergenic region reverse transcribed from *narU*⁺ RNA using primers NarU Cterm/NarZ Nterm; 5, as 4, but negative control with RNA from the *narK*⁺ strain and the primer pair NarK Cterm/NarG Nterm; 6, positive control using *narU*⁺ RNA and the primer pair NarU FORWARD/NarU REVERSE; 7, as 6, but RNA from the *narK*⁺ strain and the primer pair NarK FORWARD/NarK REVERSE.

four plasmids used for these and subsequent experiments, expression of both *narK* and *narU* was regulated by their own promoters. Plasmid pJCB901 is similar to the *narU*⁺ plasmid, pSJC901, except that it encodes NarU with a C-terminal Myc tag, for which a commercial antibody is available. Similarly, pWJ902 and pJCB902 encode NarK and NarK-Myc.

To compare the levels of accumulation of NarU-Myc and NarK-Myc during the exponential and stationary phases of growth, plasmids pJCB901 and pJCB902 were first transformed into *E. coli* strain JCB301, which is a derivative of the *narK*⁺ *narU*⁺ prototrophic strain RV in which the plasmid copy number is decreased to about 1. However, the quantity of NarU-Myc accumulated in membranes of the *pcnB* mutant was too low to detect, even by the sensitive enhanced chemiluminescence method. We therefore checked whether the expression of *narU-myc* could be amplified in a *pcnB*⁺ background without inducing artefacts due to the use of a multi-copy plasmid, and that the Myc tag did not affect NarU function. Clegg *et al.* (2002) demonstrated that there is no background rate of nitrate transport in strains that lack both NarU and NarK, irrespective whether NirC is defective or expressed. For these control experiments, the ability of

plasmids pJCB901 and pJCB902 to complement a mutant, strain JCB4520, that is defective in all of the nitrate and nitrite transport proteins (including NirC) was assessed. This strain also lacks a periplasmic nitrite reductase and is therefore suitable for assessing whether nitrite is reduced to ammonia in the cytoplasm, or accumulated in the medium. Plasmid pJCB901, like pSJC901 encoding NarU without the Myc tag, fully restored both nitrate-dependent anaerobic growth to the *narK narU nirC* triple mutant, strain JCB4520 (Fig. 2b), and nitrite accumulation in the growth medium (Fig. 2d). Both growth rates and rates of nitrite accumulation were faster than those for strain JCB4514, which expresses NarU from a single chromosomal copy of the *narU* gene. Similarly, plasmid pJCB902, encoding NarK with a C-terminal Myc tag, also restored both anaerobic, nitrate-dependent growth and nitrite accumulation (Fig. 2a, c). Rates of growth and nitrite accumulation were higher for strain JCB4516 expressing only a single chromosomal copy of *narK* than for strains transformed with multicopy plasmids, suggesting that NarK was overexpressed from these plasmids to a level that was slightly detrimental to growth.

In the *pcnB*⁺ background, despite the complete complementation of the nitrate-dependent growth defect of the

Fig. 2. Growth and nitrite accumulation by *E. coli* expressing NarK, NarK-Myc, NarU or NarU-Myc. Bacteria were grown anaerobically in 100 ml minimal salts medium supplemented with glycerol, thiamine, nitrate and 5% (v/v) LB to initiate growth. The inocula for these experiments were 2 ml of overnight aerated LB cultures. The cultures were sampled periodically as indicated. (a) Anaerobic growth of strains JCB4520 (*narK narU nirC*, ○), JCB4516 (*narK*⁺ *narU nirC*, ●), and JCB4520 transformed with a multicopy plasmid encoding NarK (pWJ902, □), or NarK-Myc (pJCB902, ■). (b) Anaerobic growth of strain JCB4520 (○), JCB4514 (*narK narU*⁺ *nirC*, ●), and JCB4520 transformed with a multicopy plasmid encoding NarU (pSJC901, □), or NarU-Myc (pJCB901, ■). (c, d) Nitrite accumulation in the culture medium by the four strains shown in (a) and (b), respectively.

narK narU double mutant, only a very faint band of NarU-Myc was detected in 200 µg of membrane proteins from bacteria harvested during the exponential phase of growth in the presence of excess nitrate (Fig. 3a, lane 1). This band was about 10-fold more intense in membranes from cultures harvested in the stationary phase of growth (Fig. 3a, lane 2). Similar results were obtained for NarU-Myc with membranes from bacteria grown anaerobically in the absence of nitrate, except that the concentration of NarU-Myc had increased at least 100-fold in the stationary-phase culture relative to the exponential-phase culture (Fig. 3b, lanes 1 and 2). In contrast, NarK was more than 1000-fold less abundant during exponential growth in the absence of nitrate than in its presence (compare lanes 3 and 4 in Fig. 3b), and the NarK concentration also decreased during the stationary phase (Fig. 3b, lane 5). In conclusion, only during the stationary phase of growth in the absence of nitrate was the concentration of NarU higher than that of NarK.

The data from the Western analysis experiments, together with the gene dosage effect seen for *narU* in the complementation experiments (Fig. 2), suggest that nitrate transport limits the rate of nitrate-dependent growth only in the absence of a functional NarK and when NarU is expressed from a single gene copy. A nitrate electrode was used to measure rates of nitrate transport and reduction by the physiological electron donor, formate, in four different strains, expressing either *narU* or *narK* from single chromosomal copies of the respective genes (JCB4014 and JCB4016, respectively), or from the multicopy plasmids pSJC901 (*narU*⁺) or pSJC902 (*narK*⁺) carried in strain JCB4520. The rate of nitrate uptake and reduction by the strain expressing only a single chromosomal copy of *narU*

was 50 ± 10 nmol nitrate reduced min⁻¹ (mg bacterial dry mass)⁻¹, significantly lower than the 80–100 nmol nitrate reduced min⁻¹ (mg bacterial dry mass)⁻¹ of the three other cultures that expressed either a single chromosomal copy of *narK*, or expressed either *narK* or *narU* from multicopy plasmids. Clegg *et al.* (2002) reported that methyl-viologen-dependent nitrate reductase activities, which indicate the activity of the cytoplasmic NarGHI complex, were similar for all four types of culture. These assays with bacterial suspensions therefore provide independent evidence that nitrate transport limits the rate of nitrate-dependent growth only in the absence of a functional NarK and when NarU is expressed from a single gene copy.

Selective advantage conferred by NarU during nutrient starvation

During anaerobic growth of *E. coli* strains JCB4014 (*narK narU*⁺) and JCB4016 (*narK*⁺ *narU*) with glycerol as the major carbon source and a growth-limiting concentration (5 mM) of nitrate as the terminal electron acceptor, both strains grew rapidly to similar final yields in either minimal salts or rich broth media. This established that these strains expressing either NarU or NarK, respectively, as the only nitrate transport protein are equally able to support nitrate-dependent anaerobic growth, and confirmed that the kanamycin resistance cassette in strain JCB4016 did not affect growth.

As *narU* is the first gene of the *narZ* operon and is also expressed preferentially during the stationary phase (Figs 2 and 3), it was possible that NarU confers a selective advantage during nutrient starvation. A chemostat was inoculated with a mixture of approximately 25 % of the *narK*⁺ strain and 75 % of the *narU*⁺ strain, and fed at a dilution rate of

Fig. 3. Western blot analysis of the accumulation of NarK-Myc and NarU-Myc during anaerobic growth. *E. coli* strain RV transformed with plasmids pJCB901 (*narU-myc*) or pJCB902 (*narK-myc*) was grown anaerobically in the presence or absence of nitrate. The cytoplasmic membrane fractions were prepared from bacteria harvested either during the middle of the exponential phase of growth, or after 16 h growth, when the cultures had entered the stationary phase. Proteins were separated by SDS-PAGE and transferred electrophoretically to a PVDF membrane. The samples were analysed by Western blotting using mouse anti-Myc antibody and horseradish-peroxidase-labelled anti-mouse IgG secondary antibody. (a). Membrane fractions of strain RV transformed with pJCB901 (*narU*⁺) from bacteria harvested in the exponential phase (lane 1) or in the stationary phase (lane 2) of growth in the presence of 20 mM nitrate. Each lane was loaded with 200 µg of membrane proteins, and the gel was exposed for 3 min. (b). Membrane fractions of strain RV transformed with pJCB901 (*narU*⁺) from bacteria harvested in the exponential phase (lane 1: 200 µg membrane protein) or in the stationary phase (lane 2: 100 µg membrane protein) during growth in the absence of nitrate. Lanes 3–5: bacteria transformed with pJCB902 (*narK*⁺) were harvested in the exponential phase of growth in the absence (lane 3: 4 µg membrane protein) or presence of nitrate (lane 4: 4 µg membrane protein). Lane 5 shows the accumulation of NarK-Myc in 4 µg membrane protein from bacteria in the stationary phase after growth in the presence of nitrate. The gel was exposed for only 10 s.

0.1 h^{-1} with minimal salts medium containing sufficient glycerol but limiting nitrate (Fig. 4). When after 48 h the proportion of the *narK*⁺ strain had increased to 90 %, the medium feed pump was switched off. After a further 24 h, the percentage of kanamycin-resistant *narK*⁺ bacteria had decreased to 50 %, confirming the selective advantage of the *NarU*⁺ strain in the absence of rapid growth. The feed was restored at a dilution rate of 0.1 h^{-1} , resulting in an increase in the percentage of *narK*⁺ bacteria to 92 % after a further 48 h. The cycle of enrichment for the *narU*⁺ bacteria was also repeated, resulting in a population of 90 % *narU*⁺ and 10 % *narK*⁺ bacteria after a further 70 h. These experiments, which were repeated three times, established that in the minimal salts medium used, a *narK*⁺ strain has a selective advantage during exponential growth, but *NarU* confers a selective advantage during nutrient starvation.

Similar results were obtained with cultures grown in a rich medium. A mixed inoculum consisting of equal numbers of the *narK*⁺ and *narU*⁺ bacteria was incubated in the fermenter without nutrient feed for 100 h, resulting in a slow decrease in the proportion of *narK*⁺ bacteria (see Supplementary Table S2, available with the online version of this paper). Subsequent growth at a dilution rate of 0.1 h^{-1} resulted in almost complete takeover of the culture by the *narK*⁺ bacteria, but this was again reversed when the feed pump was switched off. These data again revealed that a strain expressing only *narU* has a slight selective advantage during nutrient starvation compared with the *narK*⁺ bacteria, which have a considerable selective advantage during rapid growth.

Fig. 4. Competition between strains expressing either *NarK* or *NarU* during stationary- and exponential-phase growth in a minimal, defined medium. A continuous fermenter was inoculated with a mixture consisting of approximately 75 % of the *narU*⁺ strain, JCB4014 (○), and 25 % of the *narK*⁺ strain, JCB4016 (●). Steady-state growth was achieved with minimal medium supplied at a dilution rate of 0.1 h^{-1} . After 48 h, the pump was switched off. After a further 24 h, the cycle of exponential growth and starvation was repeated, as indicated. The concentration of nitrate in the feed was 20 mM, and the growth-limiting carbon source was glycerol.

Nitrate transporters *NarK* and *NarU* confer a selective advantage under different physiological conditions

Exponential-phase cultures of the same *narK*⁺ and *narU*⁺ strains mixed in an approximately 1:1 ratio were transferred into a chemostat fed with a range of different media and at different growth rates. In initial experiments, the *narK*⁺ strain rapidly out-grew the *narU*⁺ strain during the batch culture stage of the experiment. Under most growth conditions tested, the *narU*⁺ strain was also out-competed during the subsequent steady-state growth (summary data can be found in Supplementary Table S2). In each experiment, the rate of loss of the *narU*⁺ strain, JCB4014, was almost that expected for a non-growing culture, indicating that *NarK* function provided a strong selective advantage under the range of conditions tested. This included cultures in which nitrate, glycerol or glucose was the growth-limiting nutrient, the feed medium was LB or minimal salts, and at any dilution rate at or above 0.02 h^{-1} for a rich medium, or above 0.05 h^{-1} in the minimal salts medium (Supplementary Table S2). The pH of the medium remained at 6.6 ± 0.3 throughout these experiments, and the data were reproducible in replicate experiments.

These initial experiments indicated that the *narU*⁺ strain might have a selective advantage at very low growth rates. This was confirmed in subsequent experiments. First a mixed culture was grown continuously in the rich medium at a dilution rate of 0.05 h^{-1} , which resulted in an increase in the proportion of the *narK*⁺ strain JCB4016 from an initial 47 % to 89 % after 50 h (Fig. 5). The feed was then changed to the minimal salts medium, resulting in a decrease in the *narK*⁺ strain to 36 % after a further 70 h. In the final stage of the experiment, the feed was changed back to the rich medium, and the *narK*⁺ strain JCB4016 again rapidly out-competed the *narU*⁺ strain, JCB4014 (Fig. 5).

Prolonged growth in a chemostat favours strain selection, even during the period required for a new steady state to be achieved (Chao & Cox, 1983; Ferenci, 1999; Notley-McRobb *et al.*, 2002). It was essential, therefore, to establish that the apparent selective advantage of strains expressing either *narK* or *narU* was due to adaptation rather than to mutant selection during the experiment. A chemostat was inoculated with an excess (75 %) of the *narU*⁺ strain, and fed with minimal medium at a dilution rate of 0.1 h^{-1} . After 75 h, a steady state had been achieved, and the proportion of *narK*⁺ bacteria had increased to more than 80 % (Fig. 6a). The dilution rate was then decreased from 0.1 h^{-1} to 0.02 h^{-1} . After a further 175 h (which is only 6 generations), only 40 % of the bacteria were *narK*⁺. Single colonies of *narK*⁺ and *narU*⁺ bacteria from this culture were used to re-inoculate another chemostat, and the experiment was repeated to check for reproducibility. Again the *narK*⁺ bacteria were selected at the faster growth rate, but the *narU*⁺ bacteria had a selective advantage at the lower dilution rate (Fig. 6b).

Fig. 5. Effect of switching from a rich medium to a minimal medium on the selective advantage of a *narU*⁺ (○) or *narK*⁺ (●) strain during continuous slow growth. A mixed culture was grown at a constant dilution rate of 0.05 h⁻¹ in glycerol-sufficient medium. The medium was switched from LB to minimal salts at the times shown by the vertical lines. Other details are as in Fig. 4.

DISCUSSION

Two of the three nitrate reductases of *E. coli*, and also *Salmonella typhimurium*, are tightly regulated in response to changes in the growth environment, and their respective roles during anaerobic growth are clearly defined (Potter *et al.*, 1999). In contrast, the third, nitrate reductase Z, is expressed during exponential growth at such a low level that it contributes insignificantly to the total rate of nitrate reduction when either of the other two enzymes is also expressed. However, in both *E. coli* and *S. typhimurium*, its synthesis is induced under the control of RpoS during the stationary phase of growth (Chang *et al.*, 1999; Spector *et al.*, 1999). In this study, we demonstrated that NarU also accumulates during the stationary phase rather than during exponential growth, that its synthesis confers a selective advantage during nutrient starvation or very slow growth, and that *narU* is cotranscribed with *narZ*, implicating *narU* as the first gene of a five-gene polycistronic *narUZYWV* operon. Primer extension experiments failed to reveal transcripts initiated in the short intergenic region between *narU* and *narZ*. On the basis that a negative result proves nothing, the formal possibility remains that there might be a second promoter directing the transcription of the *narZYWV* genes. However, two further lines of evidence suggest that this is unlikely. First, in contrast to the potential RpoS-dependent promoter upstream of *narU*, neither a transcription terminator nor promoter elements can be recognized in the short intergenic region between *narU* and *narZ*. Secondly, although microarray data of RNA isolated from exponential-phase cultures of strain MG1655 confirmed that both *narK* and *narG* transcripts are abundant, *narU*, Z, Y, W and V are all expressed at similar very low levels (Constantinidou *et al.*, 2006). Differential expression of *narU* and *narZ* would easily have been detected in these experiments. It is therefore reasonable to suggest that NarZ also fulfils a physiological role during very slow growth, or during nutrient starvation.

Fig. 6. Adaptation rather than mutant selection as the basis for the selective advantage of the *narU*⁺ strain at very low growth rates. (a) A mixture of approximately 64% of the *narU*⁺ strain (○) and 36% of the *narK*⁺ strain (●) was inoculated into a continuous fermenter fed with nitrate-sufficient, glycerol-limited minimal medium. After 75 h at a dilution rate of 0.1 h⁻¹, the growth rate was decreased to 0.02 h⁻¹ for a further 175 h. (b) Single colonies of each strain were then purified from the mixed culture and used to inoculate another chemostat operated under the same conditions. The patterns of strain selection at the two growth rates were indistinguishable in the two experiments.

The semi-quantitative analysis of NarU and NarK accumulation in membranes during exponential growth or the stationary phase also supports the conclusion that NarU and nitrate reductase Z fulfil a physiological role during severe nutrient limitation. Although Western analysis experiments revealed that far greater quantities of NarK than NarU (in each case bearing a C-terminal Myc epitope) accumulate at any stage of growth, the concentration of NarK decreased at the end of exponential growth, but the concentration of NarU increased (Fig. 3). There was no evidence that the use of epitope-tagged proteins to detect and estimate relative quantities of NarK and NarU in bacterial membranes generated artefacts due to selective proteolysis of the Myc tag. The tagged proteins expressed from the plasmids used fully complemented mutants defective in both *narK* and *narU* (Fig. 2), even when expressed in a *pcnB* mutant in which the plasmid copy number is decreased to about one (data not shown). In contrast, in a parallel study in which amino acid substitutions have been introduced into *narU*,

the Myc tag was easily detected in proteolytic degradation products (Jia & Cole, 2005), but not from unsubstituted NarU-Myc.

Despite the very low concentration of NarU during exponential growth in the presence of nitrate, the rate of nitrate uptake and reduction by the NarU⁺ strain was over half that of the NarK⁺ strain (Fig. 4). Under these conditions, NarK is apparently present in vast excess of the concentration required for nitrate transport, and the rate of nitrate uptake and reduction must be limited either by the rate of electron transfer from the quinol pool to nitrate reductase A, or by the activity of the catalytic subunit, NarG. The alternative possibility is that NarK is a far less effective nitrate transport protein than NarU. Conversely, strains that lack NarK accumulated more nitrite in the medium when *narU* is expressed from a multi-copy plasmid than from a single chromosomal copy, indicating that NarU is limiting when *narU* is present in single copy.

Although far more NarK than NarU is present during growth in the presence of nitrate, NarU accumulates more than 100-fold during the stationary phase, even during growth in the absence of nitrate when the expression of *narK* is 100-fold lower than in its presence. Under these conditions, NarU is more abundant than NarK. This is highly significant for understanding why the *narU* operon has been conserved in enteric bacteria that must be able to survive in oxygen-limited environments, possibly in an anaerobic biofilm, where little nitrate is available and the growth rate is very low. It is exactly under such conditions that the accumulation of NarU rather than NarK will confer a selective advantage.

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