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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>JCB4011</td>
<td>RK4353 ΔnapAB ΔnarZ::Ω</td>
<td>Potter et al. (1999)</td>
</tr>
<tr>
<td>JCB4014</td>
<td>As JCB4011, but also ΔnarK</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4016</td>
<td>As JCB4011, but also ΔnarU::kan</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4018</td>
<td>JCB4011 ΔnarK ΔnarU::kan</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4514</td>
<td>As JCB4014 but also ΔnirC</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4516</td>
<td>As JCB4016, but also ΔnirC</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4518</td>
<td>As JCB4018, but also ΔnirC</td>
<td>Clegg et al. (2002)</td>
</tr>
<tr>
<td>JCB4520</td>
<td>As JCB4518, but also ΔnrfAB</td>
<td>This study</td>
</tr>
<tr>
<td>RK4353</td>
<td>ΔlacU169 arad139 rpsL gyrA non</td>
<td>Stewart (1982)</td>
</tr>
<tr>
<td>RV</td>
<td>Prototrophic F- but ΔlacZYA</td>
<td>Laboratory stocks</td>
</tr>
<tr>
<td>JCB301</td>
<td>pcnB derivative of RV</td>
<td>Clegg et al. (2002)</td>
</tr>
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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 (Bonnet 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 narUZYWX 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 narK and narU, 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 RK4333 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 ΔnirCΔnrfAB 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 pSJIC901 was constructed by cloning the PCR-generated
coding sequence of narU and approximately 1 kb of upstream DNA into the PstI site of pGEM T Easy (Clegg et al., 2002). In pCB901, 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 pCB902, 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 OD_{650} 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 mM peptatin, 1 µM leupeptin and 1 µM E-64) 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 (narU^{-}) 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 MBB 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 (narU^{-}) 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 (OD_{650} about 0-9) in Luria broth supplemented with 0-4% (v/v) glycerol and 20 mM nitrate. Total RNA was isolated using an RNAse-free spin cell mini-kit (Bioline) 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 SUPERSPECTR First-Strand Synthesis System (Invitrogen) was used to synthesize first-strand DNA from 5 µl total RNA, 5 µl 50 mM MgCl_{2}, 2 µl dithiothreitol and 1 µl RNase OUT were added. After a further 2 min at room temperature, 1 µl SUPERSPECTR 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 %, w/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, immunocomplexes were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences RPN2133).

RESULTS

Detection of transcription across the narU–narZ integron region

RT-PCR was used to determine whether narU transcription proceeds across the integron 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

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2093
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 the RpoS-dependent narZ 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.

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**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, pSJ901, 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 penB 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 penB+ 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 pSJ901 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 penB+ background, despite the complete complementation of the nitrate-dependent growth defect of the

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**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 (pSJ901, □), or NarU-Myc (pJCB901, ■). (c, d) Nitrite accumulation in the culture medium by the four strains shown in (a) and (b), respectively.
natK 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.](image-url)
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).
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* 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 *narUZYWV* 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.

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 nitrate 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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