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A single channel for nitrate uptake, nitrite export and nitrite uptake by *Escherichia coli* NarU and a role for NirC in nitrite export and uptake

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Short title: Nitrate and nitrite transport by *E. coli* NarU

Abbreviations used: Nap, periplasmic nitrate reductase; NarG and NarZ, the major and minor nitrate reductases with active sites located in the cytoplasm; Nrf, nitrite reduction by formate catalysed by the periplasmic nitrite reductase; Nir, NADH-dependent nitrite reductase; GlpT, glycerol-3-phosphate/Pi transporter; Tet, tetracycline efflux transporter; Nrt, high-affinity nitrate transporter from *Aspergillus nidulans*; MS, minimal salts; LB, Luria-Bertani broth; X-P, bromo-4-chloro indolyl phosphate; X-gal, bromo-4-chloro indolyl- β -D-galactoside.

Two related polytopic membrane proteins of the major facilitator family, NarK and NarU, catalyse nitrate uptake, nitrite export and nitrite uptake across the *Escherichia coli* cytoplasmic membrane by an unknown mechanism. A 12-helix model of NarU was constructed based upon six alkaline phosphatase and β -galactosidase fusions to NarK and the predicted hydropathy for the NarK family. Fifteen residues conserved in the NarK-NarU protein family were substituted by site-directed mutagenesis, including four residues that are essential for nitrate uptake by *Aspergillus nidulans*: arginines R87 and R303 in helices 2 and 8, and two glycines in a nitrate signature motif. Despite the wide range of substitutions studied, in no case did mutation result in loss of one biochemical function without simultaneous loss of all other functions. A NarU⁺ NirC⁺ strain grew more rapidly and accumulated nitrite more rapidly than the isogenic NarU⁺ NirC⁻ strain. Only the NirC⁺ strain consumed nitrite rapidly during the later stages of growth. Under conditions in which the rate of nitrite reduction was limited by the rate of nitrite uptake, NirC⁺ strains reduced nitrite up to 10 times more rapidly than isogenic NarU⁺ strains, indicating that both nitrite efflux and nitrite uptake are largely dependent on NirC. Isotope tracer experiments with ¹⁵N-nitrate and ¹⁴N-nitrite revealed that ¹⁵N-nitrite accumulated in the extra-cellular medium even when there was a net rate of nitrite uptake and reduction. We propose that NarU functions as a single channel for nitrate uptake and nitrite expulsion, either as a nitrate-nitrite antiporter, or more likely as a nitrate/H⁺ or nitrite/H⁺ channel.

Key words: nitrate transport, *E. coli* NarU, mutagenesis of NarU, NarU topology, ¹⁵N-nitrate experiments, nitrite uptake and export by NirC.

INTRODUCTION

During anaerobic growth, enteric bacteria reduce nitrate first to nitrite and then to ammonia. In the presence of mM concentrations of nitrate, synthesis of the periplasmic nitrate and nitrite reductases, Nap and Nrf, is substantially repressed, but a membrane-associated, cytoplasmic pathway is induced [1-6]. This requires the energy-conserving NarGHI nitrate reductase complex, which is coordinately regulated during anaerobic growth in the presence of nitrate with an energy dissipating NADH-dependent nitrite reductase, NirBD, that reduces nitrite in the cytoplasm to ammonia [4,5]. As the active site for nitrate reduction is located in the cytoplasm, nitrate must first be transported across the cytoplasmic membrane against a -180 mV proton electrochemical gradient for NarG to function [7].

Two polytopic membrane proteins of the major facilitator family, NarK and NarU, catalyse nitrate uptake into the *Escherichia coli* cytoplasm [8]. Both proteins also catalyse nitrite extrusion and nitrite uptake, but the transport mechanisms are unknown. A third protein, NirC, catalyses nitrite uptake, but it is unknown whether NirC also facilitates nitrite export [8,9]. Based upon the accumulation of nitrite as the product of nitrate reduction, it was initially proposed that NarK is a nitrate-nitrite antiporter [10,11]. An alternative suggestion that NarK is primarily a nitrite extrusion protein [12] is now known to be incorrect [8,9]. In *E. coli*, the 12 membrane helices of GlpT form a single substrate translocation pore that mediates the exchange of glycerol-3-phosphate for inorganic phosphate [13,14]. Also, the fourteen-helix TetL and TetK tetracycline efflux proteins from *Staphylococcus aureus* and *Bacillus subtilis* are believed to catalyse Na⁺, K⁺ and tetracycline antiport via a single channel that alternately imports one substrate and expels another [15]. It is therefore possible that NarK and NarU each constitutes a single channel for nitrate uptake, nitrite export, and nitrite uptake. If so, any site-directed mutation that decreases the rate of nitrate uptake should also decrease the rates of nitrite uptake and extrusion. However, this would not resolve whether nitrate uptake and nitrite export are stoichiometrically linked, for example, by an alternating access mechanism. Alternative possibilities are that NarK and NarU form a single channel for both nitrate / H⁺ symport and nitrite / H⁺ export [16], a process that does not require

strict stoichiometry between the two processes. Also possible, but less likely, is that NarK and NarU constitute two channels, one for nitrate uptake, the other for nitrite export: if so, it should be possible to separate the two functions by site-directed mutagenesis.

In preliminary experiments, we substantiated the 12-helix topology of NarK, and hence of NarU. This provided a model that predicted the location of highly conserved residues in the NarK protein family, and directed the design of site-directed mutagenesis experiments to reveal whether all known functions of NarU depend on a single transport channel. We demonstrate that NirC enhances nitrite export during nitrate-dependent growth of a strain expressing NarU, and that NirBD-dependent nitrite reduction to ammonia is enhanced by the nitrite uptake capacity of NirC. Finally ^{15}N isotope studies were designed to determine whether nitrate-nitrogen is exported into the periplasm as nitrite before it is re-imported for reduction to ammonia by the cytoplasmic nitrite reductase, NirBD.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

Strains and plasmids used during this study are listed in Table 1. Bacteria were grown aerobically at 37°C in LB (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast-extract and 5 g l⁻¹ NaCl) or anaerobically in MS medium (4.5 g l⁻¹ KH₂PO₄, 10.5 g l⁻¹ K₂HPO₄, 1 g l⁻¹ NH₄SO₄, 0.05 g l⁻¹ MgCl₂, 2.5 g l⁻¹ nutrient broth, 10 μM Na⁺-molybdate, 10 μM Na⁺ selenate and 1 ml l⁻¹ of *E. coli* sulphur-free salts [17]) supplemented as appropriate with 100 μg ml⁻¹ ampicillin and/or 50 μg ml⁻¹ kanamycin. For anaerobic growth experiments, the electron acceptor was 20 mM potassium nitrate and the primary carbon source was either 0.4% glycerol or 0.4% glucose (glucose was added as a sterile 40% w/v solution after the bulk medium had been autoclaved). The inoculum was 2% (v/v) of an exponential phase culture growing aerobically in LB. For experiments to determine the extent of nitrate-dependent growth and nitrite accumulation, bacteria were grown in 100 ml of medium in unshaken 100 ml conical flasks. Samples were removed at intervals, the optical density of the culture at 650 nm was determined, and bacteria were sedimented by centrifugation. The concentration of nitrite that had accumulated in the culture medium was then determined [8].

Mutagenesis of *narU*

Various codons in *narU* were changed using the Quikchange Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's instructions and plasmids pSJC901 and pJCB901 as the template. Specific primers were used in some of these experiments, for example, to substitute the conserved glycine residues with alanine. In many of the preliminary experiments, degenerate primers were used to substitute R87, R303 and other conserved residues by a variety of amino acids. Sequences of the primers used are available on request.

Localization of Myc-tagged proteins and Western blotting procedures

Cultures were grown anaerobically to an OD₆₅₀ of 0.5 in 1 l of MS-glucose/nitrate medium supplemented with 10% (v/v) LB. Bacteria were harvested, washed with 50 mM Tris-HCl, pH 8.0 and resuspended in 15 ml of 0.5 M sucrose, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0. Lysozyme was added to 600 μg ml⁻¹ and the suspension was incubated for 1 h at 30°C to release the periplasmic proteins [18]. The sphaeroplasts were pelleted by centrifugation for 1 h at 12,000 g, resuspended in 5 ml of 5 mM MgSO₄, 50 mM Tris-HCl, pH 8.0, and passed through a French pressure cell at 70 MPa. Unbroken bacteria and any protein in inclusion bodies were removed by centrifugation at 5,000 g for 5 min. and the cytoplasmic and membrane fractions were separated by centrifugation at 170,000 g for 1 h. Note that the membrane fraction includes inner and outer membranes and insoluble cell wall components. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-myc antibody (Invitrogen, Paisley, Scotland). Anti-

mouse IgG was obtained from TROPIX (Bedford, USA). The antibodies were detected using a chemiluminescent detection system (TROPIX Inc, Bedford, USA).

Construction of plasmids and detection of PhoA and LacZ fusion proteins

Fragments of the 5' end of the *narK* gene, including its promoter, up to the desired fusion point were amplified using PCR. The primers introduced a *Pst*I or *Eco*RI site upstream of the *narK* promoter and a *Bam*HI site at the fusion point. The *Pst*I-*Bam*HI fragments were cloned into pSK4158 [19] to create in-frame *phoA* fusions. The *Eco*RI-*Bam*HI fragments were cloned into pNM480 [20] to create in-frame *lacZ* fusions. Transformants were plated onto selective LB-agar with 40 $\mu\text{g}\cdot\text{ml}^{-1}$ bromo-4-chloro indolyl phosphate (X-P) or 40 $\mu\text{g}\cdot\text{ml}^{-1}$ bromo-4-chloro indolyl- β -D-galactoside (X-gal). Alkaline phosphatase and β -galactosidase activities were measured as described previously [21,22].

Rates of nitrate and nitrite uptake and reduction by washed bacterial suspensions

Bacteria from 1 or 2 l of an anaerobic culture were harvested by centrifugation, washed in 20 ml of Na^+/K^+ phosphate pH 7.3, resuspended in 50 mM phosphate buffer, pH 7.3, and assayed for nitrate reduction by formate using a nitrate electrode [3,8].

Rates of nitrite uptake and reduction by the cytoplasmic NirBD nitrite reductase were also determined [8,23]. For these assays, diffusion of nitrous acid across the membrane was minimised by incubating washed suspensions of bacteria with an electron donor (glucose or formate) and 0.1 mM nitrite in 50 mM MOPS buffer, pH 8.5.

Concentrations of protein and nitrite were determined using the Folin and Griess methods described previously [23].

^{15}N -nitrate reduction and recovery of ^{15}N -nitrite in cultures expressing *narU* alone, or both *narU* and *nirC*

The bacteria from 1 l of culture were harvested by centrifugation, washed and resuspended in MOPS buffer (pH 8.5). MOPS buffer (pH 8.5) was added to a 50 ml conical flask containing 2 to 5 ml of cell suspension and 1 ml of 40 % (w/v) glucose to make a final volume of 50 ml. Oxygen dissolved in this mixture was removed by sparging with nitrogen for 15 minutes. The flask was then sealed with a stopper and incubated at 30°C for 5 min. To start the assay, 250 μl of 100 mM ^{15}N -nitrate (99.5 % ^{15}N atom, Sigma-Aldrich, Poole, UK) and 250 μl of 100 mM ^{14}N -nitrite were added and the contents of the flask were mixed thoroughly. Approximately 1.5 ml samples were removed from the culture at 2 min intervals and immediately passed through a 0.2 μm Acrodisc syringe filter (Pall corporation, UK) to remove the bacteria and to stop the reduction. Approximately 1 ml of filtered medium was collected in a 1.5 ml microfuge tube and stored at -20°C.

Any nitrite in the filtrate was converted to Sudan-1, recovered by solid phase extraction and its ^{15}N content was measured by combustion and continuous flow/isotope ratio mass spectrometry (CF/IRMS) at Queen Mary, University of London [24,25]. Enrichment with ^{15}N was calculated above the background control containing MOPS buffer, 0.5 mM ^{15}N -nitrate, 0.5 mM ^{14}N -nitrite and 0.8 % (w/v) glucose. A sample or control (200 μl) was diluted with ultra-high-purity water (18 M Ω , Ultra Pure, Elga, UK) to a final volume of 50 ml and the pH was adjusted to 8.2 with 1 ml of 25 % (w/v) ammonium chloride. Any nitrite in the diluted sample was diazotized with 400 μl of 0.4% (w/v) aniline chloride (diazotization reagent) in 3 M HCl (final pH 2.0). After 5 min., 400 μl of 0.21 % (w/v) 2-naphthol (coupling reagent) in 3 M NaOH (final pH 8.0) was added. The azo dye, Sudan-1 (1-phenylazo-2-naphthol), was developed at 20°C for 10 min. The pH of this mixture was then adjusted to between 5.0 and 6.0 using approximately 75 μl of 1M citric acid. C18 Extract-Clean columns (6 ml, 500 mg; Alltech Associates Applied Science Ltd., Carnforth, UK) were placed on a 20 position solid phase extraction manifold (Alltech, UK) and conditioned with 3 ml of ethyl

acetate (analytical grade) for 1 min. The ethyl acetate was then removed under vacuum (0.35 bar) and each column was then washed (3 ml of 0.9 % w/v NaCl). Samples containing Sudan-1 were loaded onto a column and pulled through under vacuum (0.35 bar). Sudan-1 formed a discrete orange band at the top of the silica column which was then washed (50 ml of UHP water under vacuum) and eluted with 1 ml of ethyl acetate and collected in a 5 ml glass centrifuge tube. The Sudan-1 solution was dried overnight at 40-50 °C and resuspended in 200 µl of acetone, transferred to a 300 µl derivatization vial insert (2-DV; Chromacol, UK) and evaporated until approximately 20 µl of solution remained. This solution was then pipetted onto an ashed 4-mm GF/F filter disk and air-dried.

The filter disks were then placed into tin capsules for combustion and subsequent isotopic analysis by continuous flow isotope ratio mass spectrometry (Flash EA 1112 coupled to Finnigan MAT Delta^{Plus}, Thermo-Finnigan, Germany). Signals for m/z (mass charge ratio) were determined for mass 28, 29 and 30 and calibration was performed against known amounts of urea. The total accumulation of ^{15}N in nitrite was calculated as $\Delta^{15}\text{N} = \Delta^{29}\text{N}_2 + 2 \times \Delta^{30}\text{N}_2$. These experiments allowed the quantities of nitrite expelled or further reduced to ammonia to be calculated.

RESULTS

Strategies for identifying residues essential for transport functions of NarU

Five computer programmes, SPLIT, DAS, TMPRED, HMMTOP and TMHMM, all predict both NarK and NarU to be membrane proteins with 12 helices. The programme PREDTMR, however, failed to predict trans-membrane helix II within both NarK and NarU, possibly because of its lower hydrophobicity due to the presence within this helix of the arginine residue, R87 in *E. coli* NarU (Supplementary figure 1A). In preliminary experiments, we exploited the high level of expression of *narK* during anaerobic growth in the presence of nitrate to define a 12-helix model for NarU by constructing six in-frame fusions to both the β -galactosidase gene, *lacZ*, and to the alkaline phosphatase gene, *phoA*, using a plasmid-encoded *narK* gene as the template (Supplementary figure 1B). The ratio of β -galactosidase to alkaline phosphatase activities accumulated in *E. coli* strain ET8000 transformed with these plasmids was determined (Supplementary table S1). The combined data, especially the high alkaline phosphatase but low β -galactosidase activity of the fusions to P284, were consistent with the 12-helix model for NarK and NarU, but were inconsistent with models that predict fewer helices. Based on these results, a model of the topology of NarU was proposed and used to design site-directed mutagenesis experiments for functional analysis (Figure 1).

The strain used for most of the experiments in this study, JCB4520, lacks all three nitrate and nitrite transport proteins, NarK, NarU and NirC. It also lacks both Nap and NarZ, as well as the periplasmic nitrite reductase, Nrf. Consequently, this strain is unable to grow anaerobically in the presence of nitrate on a non-fermentable carbon source such as glycerol unless it is transformed with a plasmid encoding a functional nitrate transport protein. When glycerol is replaced by glucose, the strain grows as well as the parent in the absence of nitrate, but cannot augment glucose fermentation by nitrate respiration. Multicopy plasmids are available encoding NarU with or without a Myc tag (pJCB901 and pSJC901, respectively). The presence of the Myc tag has no effect on the ability of both plasmids to fully complement the defective nitrate transport phenotype of a *narK narU* double mutant [8] (reproduced in supplementary figure S2 E and F). The tagged proteins can be detected in Western blotting experiments using a commercial antibody [9]. The starting point for this study was therefore strain JCB4520 transformed with either of these plasmids (positive controls): the untransformed host provided a negative control.

NarU residues essential for membrane accumulation and all known transport functions

Moir and Wood [7] noted that few residues are totally conserved throughout cluster 6 of the major facilitator family of transport proteins that includes NarK and NarU. Site-directed mutagenesis was used to replace these residues by a range of other residues. Many of the substitutions were duplicated using two plasmid templates, plasmid pSJC901 that encodes unmodified NarU, but for which no antibody is available; and pJCB901 that encodes Myc-tagged NarU that can be detected by Western blotting using an anti-Myc antibody to detect whether the substituted protein has been incorporated into the membrane fraction. Modified plasmids were transformed into *E. coli* strain JCB4520, and the effects of each substitution on the ability of the modified NarU to support nitrate uptake required for growth on the non-fermentable carbon source, glycerol, and nitrite accumulation in the extra-cellular medium were determined. Each transformant was also grown in media supplemented with glucose and nitrate, bacteria were harvested, and rates of nitrate reduction were measured using a nitrate-specific electrode.

First we confirmed our previous report that both Myc-tagged NarU encoded by plasmid pJCB901 and NarU encoded by plasmid pSJC901 were equally able to complement all of these functions when transformed into *E. coli* strain JCB4520 (Table 2; [9]). No loss of function occurred when the conserved G414 in helix 11 was replaced by leucine, or when tyrosine 261 in helix 7 was replaced by glutamine (Table 2). Substitution of all of the 13 other conserved residues resulted in total loss of all three functions, with the following exceptions. The R87K substituted NarU supported slow nitrate-dependent growth and a slow rate of nitrite accumulation in the presence of glycerol and nitrate, although the rate of nitrate reduction by washed bacteria was insignificantly above the detection limit (Table 2; supplementary figures 2 and 3). All other substitutions in the totally conserved R87 and R303 were non-functional, consistent with the essential role of the conserved arginine residues in helices 2 and 8 of *Aspergillus nidulans* NrtA [26]. Proline, like glycine, is sometimes conserved in a membrane helix to introduce flexibility required for substrate transport through the membrane [27-29]. Although substitution of P113 by cysteine or leucine resulted in partial loss of function, this proline is clearly not essential because all of the transport activities were retained by the P113A derivative (Table 2).

Many of these and other substitutions were then introduced into NarU::Myc encoded by plasmid pJCB901 (Table 2). Without exception, similar concentrations of all of the Myc-tagged NarU proteins with substitutions at R87 or R303 were shown to accumulate in the membrane fraction at concentrations similar to unsubstituted NarU::Myc: representative data are shown in Supplementary figure 2A and 2B. Glycines G172 and G175 are part of the extended nitrate signature motif in trans-membrane helix 5 [30]. Replacement of G172 by the bulkier valine resulted in failure to accumulate and hence, loss of function (Table 2). However, nitrate uptake and nitrite export functions were retained when glycine was replaced by alanine (Table 2 and supplementary figures 4A and 4C), indicating that a small residue was essential at this point of helix 5 for the correct folding and accumulation of NarU in the membrane. By contrast, substitution of G175 by either serine or alanine resulted in loss of function (supplementary Figures 4B and 4D), despite the fact that the G175A protein was readily detected within the membrane by Western analysis (Supplementary figure 5). Replacement of G139 in helix 4 by arginine or glutamate also resulted in complete loss of function: incorporation of the G139I protein into the membrane was confirmed (Supplementary figure 5). G266 in helix 7 and G405 in helix 11 were totally essential for function even when these glycines were replaced by alanine (Table 2), and none of the variants G266T, P or A, or G405 L or A, were detected in membrane preparations by Western analysis (Supplementary Figure 5). Replacement of G162 by either serine or alanine or G307 by leucine resulted in complete loss of function due to failure to be incorporated into the membrane (Table 2 & supplementary Figure 5), suggesting that these residues might be conserved to introduce tight turns in short cytoplasmic loops between helices 4 and 5 and 8 and 9, respectively. In contrast, substitution of G99 by threonine resulted in only partial loss of function, but no loss of transport activity resulted from the G99A substitution.

In summary, results of the site-directed mutagenesis experiments were readily interpreted on the basis of the predicted topology of NarU (Fig. 1), and they revealed for the first time that the

majority, but not all, of the highly conserved residues of NarU are for various reasons important or essential for all four known functions of NarU. This indicated that a single transport channel is likely to be used for nitrate uptake and nitrite extrusion.

NirC stimulates growth rate, nitrite extrusion, and nitrite uptake during anaerobic growth of a NarU⁺ strain in the presence of nitrate

Having demonstrated that NarU can catalyse nitrate uptake, nitrite export, and nitrite uptake, we next investigated the physiological role of NirC during anaerobic growth in the presence of nitrate. During nitrate-dependent growth on the non-fermentable carbon source, glycerol, the NarU⁺ NirC⁺ strain, JCB4014, grew more rapidly and accumulated nitrite more rapidly than the isogenic NarU⁺ NirC⁻ strain, JCB4514 (Figure 2A, B). By the stationary phase of growth, more than 70% of the nitrate added to the medium had been accumulated as extracellular nitrite. The rate of growth of the NarU⁺ NirC⁺ strain in the presence of glucose was also more rapid than that of the NarU⁺ NirC⁻ strain, JCB4514, (Fig. 2C), and the maximum concentration of nitrite accumulated in the extracellular medium was again 70% of the initial concentration of nitrate (Fig. 2D). However, only the NirC⁺ strain consumed nitrite rapidly during the later stages of growth (Fig. 2D). These results indicated that NirC can facilitate both extrusion of nitrite generated during nitrate reduction, and uptake of nitrite.

To demonstrate directly that NirC can catalyse more rapid nitrite uptake than NarU, strains expressing various combinations of *narU* and *nirC* were harvested after growth in the presence of glucose and nitrate. Rates of nitrite uptake and reduction were determined under conditions in which rates of nitrous acid diffusion into the bacteria were minimized, but the rate of nitrite reduction by the cytoplasmic nitrite reductase, NirB, was limited by the rate of nitrite uptake (Fig. 3; [9]). Rates of nitrite reduction by the NarU⁺ NirC⁺ and NarU⁻ NirC⁺ strains, JCB4014 and JCB4018 were in the range 45 to 65 nmol of nitrite reduced (mg dry bacteria)⁻¹. min⁻¹ (Fig. 3). Nitrite was reduced at only a very low rate (<2 nmol of nitrite reduced (mg dry bacteria)⁻¹. min⁻¹) by strain JCB4520 that lacks both NarU and NirC, but more rapidly by the NarU⁺ NirC⁻ strain, JCB4514 (6 nmol of nitrite reduced (mg dry bacteria)⁻¹. min⁻¹). All of the substituted NarU proteins encoded by plasmid pSJC901 and many with substitutions in NarU::Myc encoded by plasmid pJCB901 were also checked for ability to restore nitrite uptake and reduction to strain JCB4520. There was complete correlation between ability to restore nitrite uptake and reduction, nitrate-dependent growth and nitrite export in the presence of glycerol, and rates of nitrate reduction after growth in the presence of glucose (data not shown).

These experiments established that both nitrite efflux and nitrite uptake are largely dependent on NirC. Conversely, in the absence of NirC, the ability of NarU to catalyse nitrite export rather than nitrate uptake limits the rate of nitrate uptake and growth. This raised the question whether nitrite formed during nitrate reduction is exported by NarU and NirC before it is re-imported for reduction in the cytoplasm by NirBD.

Direct evidence from ¹⁵N isotope experiments that the nitrate-N is exported as nitrite before it is re-imported for reduction to ammonia

The combined data from the site-directed mutagenesis experiments and biochemical experiments with strains expressing different combinations of NarU and NirC indicated that NarU is primarily a nitrate uptake protein that can also catalyse a much slower rate of nitrite transport (Figure 3 and reference [8]). Nitrate labelled with ¹⁵N was used to demonstrate that the nitrogen atom of nitrate is first expelled as nitrite before it is re-imported for reduction to ammonia.

Bacteria that had been grown in minimal medium supplemented with glucose and nitrate were harvested, washed, and incubated with 0.5 mM ¹⁵N-nitrate, 0.5 mM ¹⁴N-nitrite and 0.8% glucose. To minimise the diffusion of ¹⁴N-HNO₂ across the membrane, MOPS buffer pH 8.5 was used for these experiments [23]. The extra-cellular concentrations of nitrate, ¹⁵N-nitrite and total

nitrite concentration (^{14}N -nitrite plus ^{15}N -nitrite determined by direct chemical assay) during incubation at 37°C were measured, and the concentration of ^{14}N -nitrite was calculated by difference (Figure 4).

There was an immediate decrease in total nitrite concentration when the $\text{NarU}^+ \text{NirC}^+$ strain JCB4014 was incubated with the equimolar mixture of nitrate and nitrite (Figure 4A). Despite this net decrease, ^{15}N -nitrite accumulated in the buffer at a rate of $19.6 \text{ nM min}^{-1} (\text{mg of dry bacteria})^{-1}$. This result suggested that 37% of the ^{15}N -nitrite produced was expelled from the cytoplasm, while 63% was reduced to ammonia at a rate of $33.4 \text{ nM min}^{-1} (\text{mg of dry bacteria})^{-1}$. The total rate of nitrite reduction by the $\text{NarU}^+ \text{NirC}^+$ strain was $66.4 \text{ nM min}^{-1} (\text{mg dry bacteria})^{-1}$, which was calculated as 63% of the rate of nitrate reduction plus the net rate of decrease in extracellular nitrite concentration ($33 \text{ nM min}^{-1} (\text{mg dry bacteria})^{-1}$).

In contrast, during the initial 10 minutes of incubation of the $\text{NarU}^+ \text{NirC}^-$ strain JCB4514, the concentration of ^{15}N nitrate decreased at a rate of $17.6 \text{ nM min}^{-1} (\text{mg dry bacteria})^{-1}$, but extracellular ^{15}N nitrite accumulated in the external medium at a rate of $15.0 \text{ nM min}^{-1} (\text{mg of dry bacteria})^{-1}$ (Fig. 4B). Thus, 87% of the nitrate reduced had accumulated extracellularly as $^{15}\text{NO}_2^-$. Therefore, despite the presence of a highly active NirBD nitrite reductase, only 13% of the nitrate-N had been reduced to ammonia at a rate of $2.2 \text{ nM min}^{-1} (\text{mg of dry bacteria})^{-1}$. There was also a net increase in the total extracellular nitrite concentration during this period. Results from the two groups of experiments clearly showed that most of the nitrate-nitrogen was expelled as ^{15}N -nitrite before it could be re-imported via NirC for reduction in the cytoplasm to ammonia.

DISCUSSION

Surprisingly little is known about how nitrate is transported across bacterial membranes. Moir and Wood [7] noted that multi-component, ATP-dependent transport complexes are common in organisms that assimilate nitrate. In contrast, the NarK homologues of the major facilitator family of transport proteins are more common in denitrifying bacteria, or in bacteria that reduce nitrate to ammonia during anaerobic nitrate respiration. They also noted that the NarK family fall into three distinct groups, two sub-clusters of bacterial nitrate transporters, and the more distantly related fungal nitrate transporters. In preliminary experiments, the high level of expression of *E. coli narK* was exploited to confirm that NarK, and therefore also NarU, are similar to *A. nidulans* NrtA, with 12 membrane-spanning helices and N- and C-termini located in the cytoplasm. We also showed that R89 is essential for nitrate transport by NarK [31]. However, during growth in the presence of nitrate, so much NarK accumulates that any residual transport activity following substitution of other amino acids was sufficient to support nitrate-dependent anaerobic growth [9]. Substitutions engineered into a plasmid-encoded *narU* gene therefore provided a more sensitive and reliable experimental system to investigate the effects of substituting highly-conserved residues on nitrate and nitrite transport. Despite the very wide range of substitutions studied, in no case did mutation result in loss of one transport function without simultaneous loss of all other functions.

Irrespective whether the functional nitrate transporter available was NarK or NarU, in this and previous work, nitrite accumulated quantitatively in the medium when washed bacterial suspensions were incubated in the presence of nitrate and formate, which cannot donate electrons to the cytoplasmic NirBD nitrite reductase [8,9]. However, during growth in the presence of glucose and nitrate, at least some of the nitrate imported was reduced to ammonia rather than accumulated in the surrounding medium. A critical question is therefore whether nitrite generated during nitrate reduction is immediately reduced in the cytoplasm by NirBD to ammonia. Alternatively, if NarK and NarU are simply nitrate / nitrite antiporters, this would require nitrite to be exported at least into the periplasm before it can subsequently be re-imported via NarK, NarU or NirC for reduction by NirBD. That the latter possibility is correct was shown both by physiological experiments in which the effects of NirC on nitrite accumulation and reduction were investigated, and from the results of experiments in which ^{15}N -nitrate and ^{14}N -nitrite were reduced simultaneously in the presence or absence of a functional NirC. The combined data from these experiments revealed that NirC is up to

10-fold more active than NarK or NarU in nitrite uptake for subsequent reduction in the cytoplasm by NirBD. Despite a net rate of nitrite uptake and reduction by the NirC⁺ strain, ¹⁵N-nitrite still accumulated in the extra-cellular medium. We conclude that nitrate uptake and reduction is mechanistically linked to nitrite expulsion.

Some evidence that nitrate uptake by *E. coli* strains expressing either only *narK* or only *narU* is sensitive to inhibition by chemicals that collapse the membrane potential was obtained previously using a proteoliposome system [12]. Furthermore, some bacteria synthesize two types of nitrate transport protein that fall into the two different sub-clusters of the NarK protein family [7,32]. Wood et al [16] proposed that one sub-cluster catalyses nitrate-nitrite antiport, the other nitrate / H⁺ symport. We suggest that NarU constitutes a channel for both nitrate / H⁺ symport and nitrite / H⁺ export, processes that do not require strict stoichiometry between uptake and export, and accounts for how nitrate uptake can be initiated in the absence of pre-formed nitrite.

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Figure Legends

Figure 1 Location of conserved residues in *E. coli* NarU

The predicted locations of 15 NarU residues that are highly conserved throughout cluster 6 of the major facilitator family of transport proteins are shown in black, and are listed below the figure in order from R87 in trans-membrane helix 2 to G414 in trans-membrane helix 11. Each of these residues was substituted in the site-directed mutagenesis experiments.

Figure 2 Physiological role of NirC in nitrate-dependent growth and nitrite accumulation

An isogenic set of strains expressing all combinations of *narU* and *nirC* were grown anaerobically in the presence of 20 mM nitrate, and the optical density and concentration of nitrite in the culture medium were determined at intervals. The strains used in these experiments and their relevant genotypes are JCB4014 *narK narU⁺ nirC⁺*; JCB4018 *narK narU nirC⁺*; JCB4514 *narK narU⁺ nirC*; and JCB4518 *narK narU nirC*. (A) and (B). The non-fermentable carbon source, glycerol was used for these experiments. As nitrate was the only available terminal electron acceptor, strains lacking both NarK and NarU are unable to grow in this medium. (C) and (D). Growth and nitrite accumulation of strains able to reduce nitrate in the presence of glucose and 20 mM nitrate. Note that, irrespective of the carbon source, both growth and nitrite export into the medium were stimulated by the presence of a functional NirC.

Figure 3 Rates of nitrite uptake and reduction dependent upon NarU and NirC

The isogenic set of strains expressing all combinations of *narU* and *nirC* were grown in the presence of glucose and nitrate, harvested in the late exponential phase of growth, washed with MOPS buffer (pH 8.5) and resuspended in the same buffer. Low rates of nitrite reduction at pH 8.5 determined in the presence of formate were deducted from the rate in the presence of glucose to give the NirBD-dependent rate of nitrite uptake and reduction: these rates were typically 1 – 2 nmol of nitrite reduced (mg dry bacteria)⁻¹ min⁻¹. Note that under the conditions used for these experiments, the rate of nitrite reduction is determined by the rate of nitrite uptake via NarU or NirC [8,23].

Figure 4 ¹⁵N isotope experiments to demonstrate NarU-dependent export of nitrate-N as nitrite before it is re-imported for reduction to ammonia

The NarU⁺ NirC⁺ *E. coli* strain JCB4014 and the isogenic NarU⁺ NirC⁻ strain JCB4514 were grown in MS medium supplemented with glucose and nitrate, harvested, washed and resuspended in MOPS buffer, pH 8.5. These suspensions were incubated with 0.5 mM ¹⁵N-nitrate, 0.5 mM ¹⁴N-nitrite and 0.8% glucose. The concentrations of nitrate, ¹⁵N-nitrite and total nitrite (¹⁴N-nitrite plus ¹⁵N-nitrite measured by direct chemical assay) were determined at intervals. The concentration of ¹⁴N-nitrite was calculated as the difference between the concentrations of total nitrite and ¹⁵N-nitrite. (A) The NarU⁺ NirC⁺ strain JCB4014 at a concentration of 0.96 mg dry mass ml⁻¹. (B) The NarU⁺ NirC⁻ strain, JCB4514 at a concentration of 1.9 mg dry mass ml⁻¹.

Table 1 Strains and plasmids used in this work

Name	Genotype	Source
Strains		
JCB4514	RK4353 Δ <i>napA-B</i> Δ <i>narZ::\Omega</i> Δ <i>narK::tet</i> Δ <i>nirC::cam</i>	[9]
JCB4518	RK4353 Δ <i>napA-B</i> Δ <i>narZ::\Omega</i> Δ <i>narK::tet</i> Δ <i>narU::kan</i> Δ <i>nirC::cam</i>	[9]
JCB4014	RK4353 Δ <i>napA-B</i> Δ <i>narZ::\Omega</i> Δ <i>narK::tet</i>	[9]
JCB4018	RK4353 Δ <i>napA-B</i> Δ <i>narZ::\Omega</i> Δ <i>narK::tet</i> Δ <i>narU::kan</i>	[9]
JCB4520	RK4353 Δ <i>napA-B</i> Δ <i>narZ::\Omega</i> Δ <i>narK::tet</i> Δ <i>narU::kan</i> Δ <i>nirC::cam</i> Δ <i>nrfA::chl</i>	[9]
ET8000	<i>rbs lacZ::IS1 gyrA hutC</i> κ	[33]
Plasmids		
pSJC901	3.5 kb PCR fragment containing the <i>narU</i> gene, ligated into pGEM® T Easy	[9]
pJCB901	2.5 kb PCR fragment containing the <i>narU</i> gene and a myc tag, ligated into pGEM® T Easy	[9]
pSK4158	Chl ^R PhoA fusion vector	[19]
pNM480	Carb ^R LacZ fusion vector	[20]

Table 2 Nitrate-dependent growth and nitrite accumulation by strain JCB4520^a transformed with plasmids encoding NarU with mutations at 13 conserved residues

Plasmid	Predicted location of mutated residue ^b	Growth on glycerol + nitrate	Nitrite accumulation during growth on glycerol + nitrate	Detection of Myc-tagged NarU after growth with glucose + nitrate
pGEM-TEasy ^c		-	-	NA ^d
pSJC901 control ^f		+++	+++	NA ^d
G99A	CL2/3	+++	+++	NA ^d
P113C	TM3	+	+	NA ^d
P113L	TM3	+	+	NA ^d
P113A	TM3	+++	+++	NA ^d
G139E	TM4	-	-	NA ^d
G172A	TM5	+++	+++	NA ^d
G175S	TM5	-	-	NA ^d
Y261N	TM7	+++	+++	NA ^d
G405V	TM11	-	-	NA ^d
G414L	TM11	+++	+++	NA ^d
pJCB901 control ^g	No mutation	+++	+++	+
G99T-myc	CL2/3	+	+	NA ^e
G139I-myc	TM4	-	-	+
F145E-myc	TM4	-	-	-
F145W-myc	TM4	-	-	+
G162S-myc	CL4/5	-	-	-
G162A-myc	CL4/5	-	-	-
G172V-myc	TM5	-	-	-
G175A-myc	TM5	-	-	+
G266T-myc	TM7	-	-	-
G266P-myc	TM7	-	-	-
G266A-myc	TM7	-	-	-
G307L-myc	CL8/9	-	-	+
D311G-myc	CL8/9	-	-	+
D311K-myc	CL8/9	-	-	-
G405L-myc	TM11	-	-	-
G405A-myc	TM11	-	-	-

a. Strain JCB4520, deleted in *narK*, *narU* and *nirC*, was transformed with plasmids expressing mutated derivatives of NarU. Nitrate-dependent anaerobic growth with glycerol as carbon source and extrusion of nitrite were dependent upon nitrate and nitrite transport by the plasmid-encoded NarU protein.

b. Predicted locations are based on the proposed topology of NarU shown in Figure 1. TM3, transmembrane region 3; CL2/3, cytoplasmic loop between TM 2 and 3.

c. pGEM-TEasy was the vector used to clone *narU*.

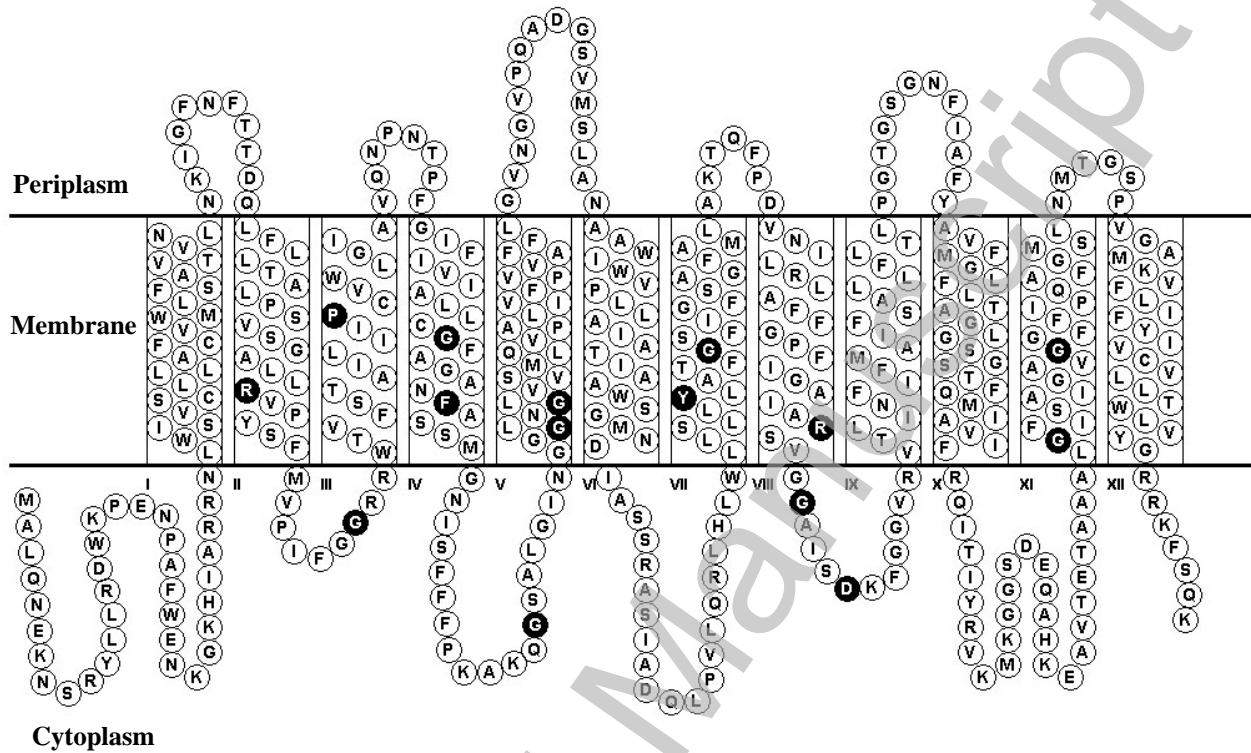
d. NA: not applicable because no Myc-tag was attached.

e. NA: not assayed because the substituted protein was functional, and therefore must have been incorporated into the membrane.

f. Strain JCB4520 transformed with plasmid pSJC901 encoding wild-type NarU.

g. Strain JCB4520 transformed with plasmid pJCB901 encoding wild-type NarU with a Myc tag.

Figure 1



Order of conserved residues: R87, G99, P113, G139, F145, G162, G172, G175, Y261, G266, R303, G307, D311, G405 and G414.

Figure 2

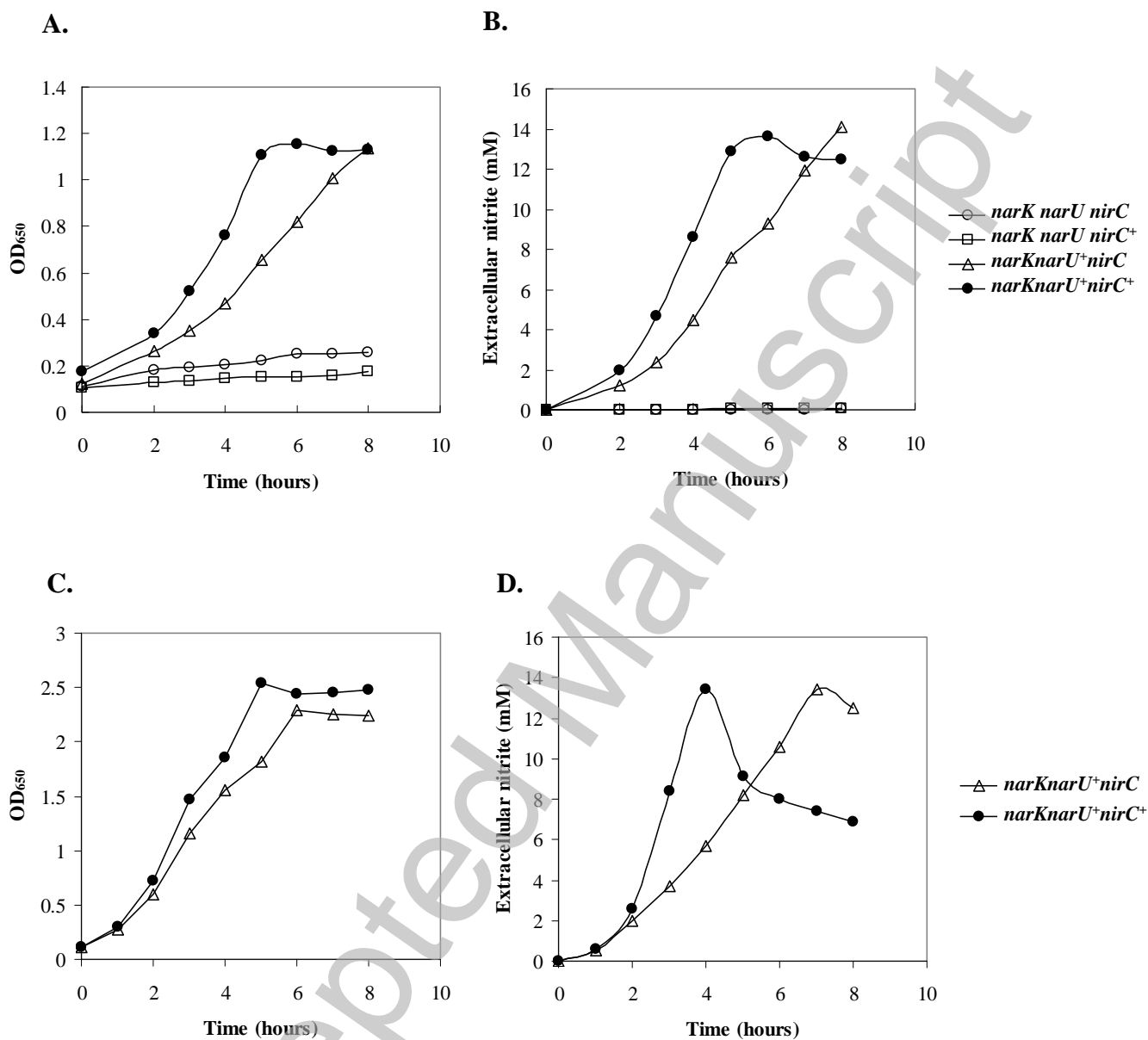


Figure 3

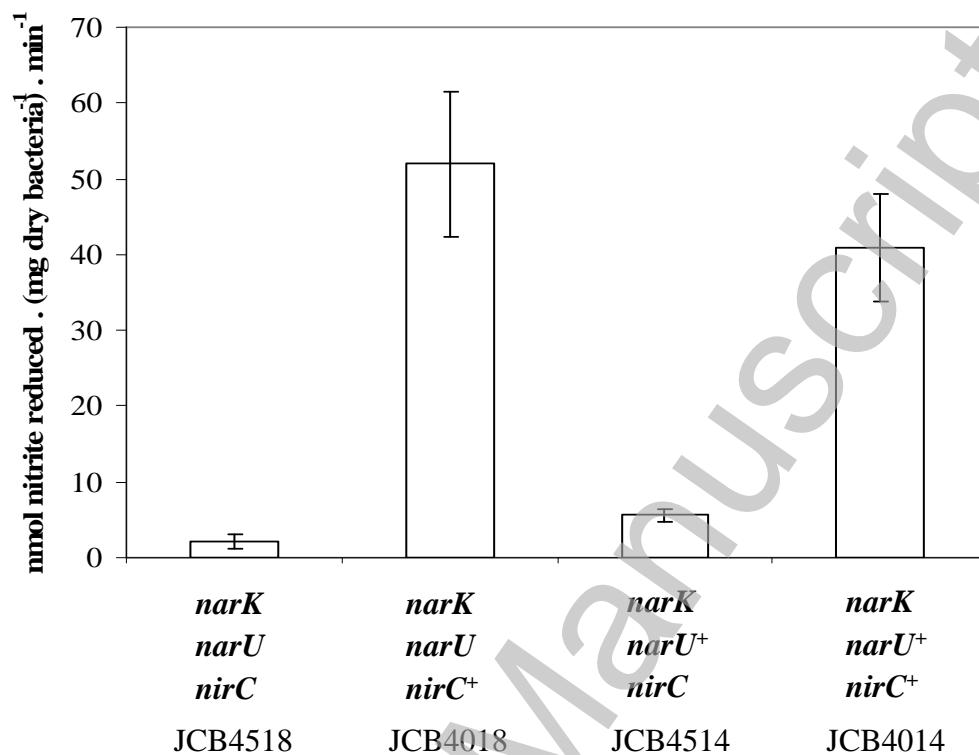
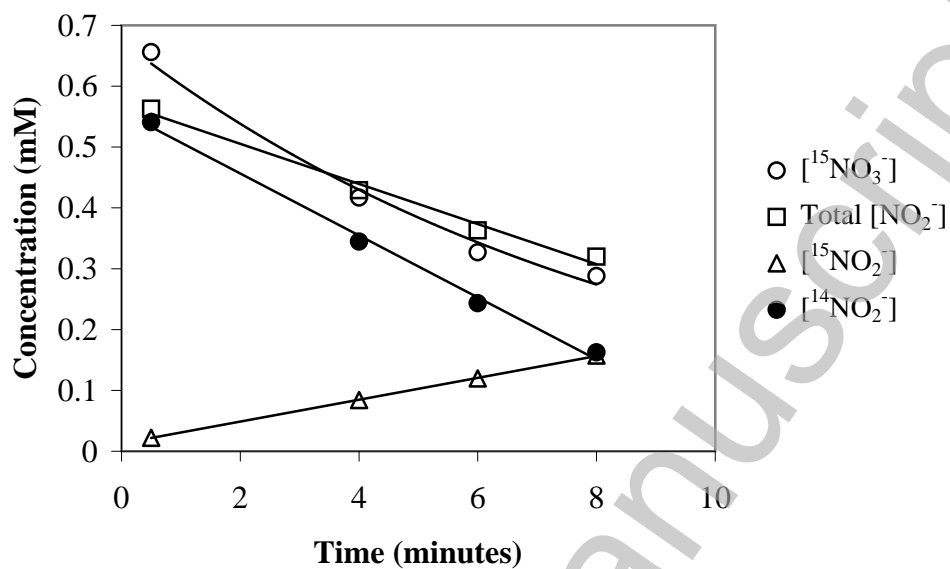


Figure 4

A. Strain JCB4014 *narKnarU⁺nirC⁺*B. Strain JCB4514 *narKnarU⁺nirC⁻*