Mutational Analysis of the Respiratory Nitrate Transporter NarK2 of *Mycobacterium tuberculosis*

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Abstract

*Mycobacterium tuberculosis* induces nitrate reductase activity in response to decreasing oxygen levels. This is due to regulation of both the transcription and the activity of the nitrate transporter NarK2. A model of NarK2 structure is proposed containing 12 membrane spanning regions consistent with other members of the major facilitator superfamily. The role of the proton gradient was determined by exposing *M. tuberculosis* to uncouplers. Nitrite production decreased indicating that the importation of nitrate involved an H⁺/nitrate symporter. The addition of nitrite before nitrate had no effect, suggesting no role for a nitrate/nitrite antiporter. In addition the NarK2 knockout mutant showed no defect in nitrite export. NarK2 is proposed to be a Type I H⁺/nitrate symporter. Site directed mutagenesis was performed changing 23 amino acids of NarK2. This allowed the identification of important regions and amino acids of this transporter. Five of these mutants were inactive for nitrate transport, seven produced reduced activity and eleven mutants retained wild type activity. NarK2 is inactivated in the presence of oxygen by an unknown mechanism. However none of the mutants, including those with mutated cysteines, were altered in their response to oxygen levels. The assimilatory nitrate transporter NasA of *Bacillus subtilis* was expressed in the *M. tuberculosis* NarK2 mutant. It remained active during aerobic incubation showing that the point of oxygen control is NarK2.

Introduction

Nitrate can serve as a terminal electron acceptor as well as a source of nitrogen for many bacteria. The first step for either of these processes involves the active transportation of nitrate across a membrane into the cell where it is reduced to nitrite. Nitrate, a charged molecule, must rely on a transporter to be efficiently imported. Nitrate transport has been studied in a variety of microbes. Transport during nitrate respiration has mostly focused on gram negative bacteria such as *E. coli* and *Paracoccus sp*. For the purpose of assimilation *Aspergillus nidulans* has been the model organism. Nitrate is a major source of nitrogen for higher plants including many crops. Thus the topic is of economic and medical importance.

The major facilitator superfamily consists of transporters found in all kingdoms of life [1]. Family 8 (TC 2.A.1.8) of this superfamily comprises the nitrate/nitrite porter proteins (NNP). Phylogenetic analysis of the bacterial NNPs further identified two subgroups [2,3]. Type I were proposed to be H⁺/nitrate symporter. Phylogenetic analysis of the bacterial NNPs further identified two subgroups [2,3]. Type I were proposed to be H⁺/nitrate symporter. Site directed mutagenesis was performed changing 23 amino acids of NarK2. This allowed the identification of important regions and amino acids of this transporter. Five of these mutants were inactive for nitrate transport, seven produced reduced activity and eleven mutants retained wild type activity. NarK2 is inactivated in the presence of oxygen by an unknown mechanism. However none of the mutants, including those with mutated cysteines, were altered in their response to oxygen levels. The assimilatory nitrate transporter NasA of *Bacillus subtilis* was expressed in the *M. tuberculosis* NarK2 mutant. It remained active during aerobic incubation showing that the point of oxygen control is NarK2.

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**Summary**

Nitrate can be a terminal electron acceptor for respiration and a source of nitrogen for many bacteria. The first step for either of these processes involves the active transportation of nitrate across a membrane into the cell where it is reduced to nitrite. Nitrate transport has been studied in a variety of microbes, including gram negative bacteria such as *E. coli* and *Paracoccus sp.*. For the purpose of assimilation, *Aspergillus nidulans* has been the model organism. Nitrate is a major source of nitrogen for higher plants including many crops. Thus the topic is of economic and medical importance.

The major facilitator superfamily consists of transporters found in all kingdoms of life. Family 8 (TC 2.A.1.8) of this superfamily comprises the nitrate/nitrite porter proteins (NNP). Phylogenetic analysis of the bacterial NNPs further identified two subgroups. Type I were proposed to be a H⁺/nitrate symporter. Site directed mutagenesis was performed changing 23 amino acids of NarK2. This allowed the identification of important regions and amino acids of this transporter. Five of these mutants were inactive for nitrate transport, seven produced reduced activity and eleven mutants retained wild type activity. NarK2 is inactivated in the presence of oxygen by an unknown mechanism. However, none of the mutants, including those with mutated cysteines, were altered in their response to oxygen levels. The assimilatory nitrate transporter NasA of *Bacillus subtilis* was expressed in the *M. tuberculosis* NarK2 mutant. It remained active during aerobic incubation showing that the point of oxygen control is NarK2.
respiration is the transport of nitrate. Therefore we set out to further characterize the function of NarK2. To gain insight into the mechanism of nitrate transport NarK2 was analyzed for both H+/NO₃⁻ symporter, and nitrate/nitrite antiporter activity. Site-directed mutagenesis of conserved amino acids identified important regions and residues of the protein.

**Materials and Methods**

**Culture Conditions**

*M. tuberculosis* H37Rv was grown in Dubos Tween-albumin broth (DTA, Difco, Detroit, MI). Growth was monitored by measuring the OD₅₆₀ in a Coleman model 35 spectrophotometer (Coleman Instruments, Maywood, IL). Cultures were started with an initial density of 2.5×10⁶ cells/ml. Aerobic cultures were incubated at 37°C on a model G24 rotary shaker-incubator at a speed of 225 rpm [New Brunswick Scientific Co. Inc, Edison, NJ].

For microaerobic and anaerobic cultures the Wayne model was used with culture tubes sealed with Wheaton red rubber septum caps (Fisher Scientific, Pittsburgh, PA) and wrapped with parafilm [8]. After approximately 67 hrs growth stopped and cultures of *M. tuberculosis* entered the microaerobic nonreplicating persistent state I (NRP-1). Fully anaerobic NRP-2 was reached after roughly 200 hrs of incubation.

**Treatment with Protonophores**

NRP-1 cultures, containing approximately 10⁶ cells/ml were opened and pooled. Aerobic cultures were diluted in DTA to the same cell concentration based on optical density (OD₅₆₀ 0.1 = 6.25×10⁹ CFU/ml). NaNO₃ was added to 5 mM. The uncouplers (Sigma, St. Louis, MO) in water were added. 2,4-dinitrophenol (DNP) was used at 1 mM, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was at 200 μM. Cultures were dispensed into tubes in triplicate. For aerobic conditions tubes were loosely capped and incubated with shaking. For anaerobic conditions cultures were filled to the top and Oxystar was added to remove oxygen (Oxystar Inc Mansfield, OH). For some tubes NaNO₂ and Oxystar were added and then incubated at 37°C for 4 hrs before the nitrate was added. Nitrite concentrations were measured at 2.5 hrs intervals. To measure oxygen utilization methylene blue was added to 0.0003% and the concentrations were measured at 2.5 hrs intervals. To measure resistance for hygromycin. The nasA insertion was verified by Southern analysis.

**Detection of Nitrite in Cells**

Cultures of RVW3 (ΔnarK2) were grown to NRP-1 (115 h). The cells were centrifuged and resuspended in the same volume of DTA with 5 mM NaNO₃ also containing 5 mM benzyl viologen (where indicated) at either pH 6.7 or 8.5. 500 μl of culture was transferred to 0.65 ml graduated microtubes (in triplicate). The reaction was initiated with the addition of 120 μl of 60 mM dithionite in 10 mM NaOH which reduced the benzyl viologen. To a control set of tubes 120 μl of 10 mM NaOH was added (oxidized benzyl viologen samples). After 1.5 hrs incubation at 37°C the tubes were centrifuged. The medium was removed while the cells were treated for 10 min in 1 M HCl. Nitrite levels were then determined in both the medium and cells fractions.

RVW3 pNarK2 and RVW3 pNasA were grown to NRP-1 in the Wayne model. They were treated as above except instead of benzyl viologen, Oxystar was added. After 3 hrs cells were pelleted by centrifugation and the medium carefully removed.

**Cloning of nasA in *M. Tuberculosis***

The nasA of *Bacillus subtillis* was amplified with the primers pNasA-F and pNasA-R (Table S1). Two reactions were run and the 1510 bp fragment from each was cloned and sequenced. These were compared to the published nasA sequence [22]. Both of the new sequences differed from the published nasA sequence by the same base. This change would result in a NasA protein that was 20 amino acids longer than previous reported. The longer NasA was more similar to NasA sequences from other *Bacillus* species and was verified as correct by additional PCR and sequencing.

The promoterless nasA was inserted into the integrating plasmid pMP102 in front of the narK2 promoter by digesting both plasmids with EcoRI and KpnI [15]. A hygromycin resistance marker was inserted into the Smal site to make pNasA. This plasmid was electroporated into *M. tuberculosis* RVW3 (ΔnarK2) with selection for resistance for hygromycin. The nasA insertion was verified by Southern analysis.

**Mutagenesis of narK2**

All primers were from Sigma-Aldrich (Table S1). The narK2 complementing plasmid pNarK2 was used for mutagenesis [23]. This plasmid was used for site directed mutagenesis using the QuickChange® II XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). After mutagenesis the plasmids were transformed into E. coli with selection for gentamycin resistance. Plasmids containing the mutation were identified by sequencing. Each plasmid was then electroporated into *M. tuberculosis* RVW3 (ΔnarK2) followed by selection for gentamycin resistance [15]. Southern analysis was performed to verify the presence of both the inactive wild type gene and the presence of the mutated gene. PCR was use to amplify the mutated narK2 using primers p173 and p174 (Table S1) and the product sequenced to verify the presence of the correct mutation.

To measure nitrite production cultures were grown in DTA with 5 mM NaNO₃ for 115 hrs in shaking cultures (aerobic), 115 hrs in the Wayne model (microaerobic NRP-1) or 255 hrs in the Wayne model (anaerobic NRP-1) or 255 hrs in the Wayne model (anaerobic NRP-2). Aerobic cultures were in mid-logarithmic phase (OD₅₆₀=0.4) whereas NRP-1 cultures were hypoxic for approximately 48 hrs [8]. Samples were removed and nitrite concentrations determined by the Griess reaction.

**Results**

**Role of the Proton Gradient**

During growth in DTA medium *M. tuberculosis* reduces nitrate to nitrite but does not reduce nitrite further. The nitrite accumulates in the extracellular medium and can be used as a sensitive method to monitor the combined activity of nitrate transport and its reduction. Aerobic cultures of *M. tuberculosis* do not expresses narK2 and have only a low level of nitrate reductase activity resulting from diffusion of nitrate into the cell [15,17]. In the Wayne model for dormancy narK2 is induced at the beginning of microaerobic NRP-1 and maintained at a high level in anaerobic NRP-2 [15,16].

Uncouplers were used to determine if the proton gradient plays a role in NarK2-mediated import of nitrate. The effect of two different uncouplers on the nitrate reductase enzyme was first measured. FCCP and DNP were added to aerobic cultures of *M. tuberculosis* and nitrite levels measured. FCCP and DNP had little effect on nitrite production indicating no role for the proton gradient in the reduction of nitrate under aerobic conditions (Figure 1A).
The effect of each of these compounds on nitrite production by hypoxic narK2-expressing cultures was next determined. *M. tuberculosis* was grown to NRP-1 and both nitrate and an uncoupler were added. FCCP and DNP both decreased the rate of nitrite production indicating a role for the protein gradient in nitrate transport by NarK2 (Figure 1B). In aerobic and anaerobic cultures preincubation with 50 µM NO$_3^-$ did not alter the effect of the uncouplers. A decrease in the proton gradient could also reduce glucose uptake which could limit energy production. To verify that the decreased nitrite production is due to the inhibition of nitrate transport and not of metabolism, oxygen utilization was measured. The rate of methylene blue decolorization was used to monitor oxygen levels in the presence of each uncoupler (Figure 1C). No inhibition of respiration was seen.

**NarK2 does not Function as a Nitrate/nitrite Antiporter**

To determine if NarK2 functions as a nitrate/nitrite antiporter, the effect of nitrite on the initial rate of nitrate reduction was determined. Upon the addition of nitrate NNP antiporters have low activity until sufficient nitrate diffuses into the cell to provide nitrite for activity. This lag can be eliminated by providing nitrite in advance [4]. *M. tuberculosis* was grown in the Wayne model and cultures were aliquoted to tubes with either no addition, or nitrite at either 50 µM or 100 µM. These samples were incubated anaerobically with Oxyrase at 37 C. After 4 hours of pretreatment to allow nitrite to enter the cells, nitrate was injected and nitrite concentrations were determined 1 and 2 hours after the addition (Figure 2). Without pretreatment cells produced nitrite at a rate of 51 µM/hr. With the 50 µM NaNO$_2$ pretreatment the rate was similar, 55 µM/hr, and with 100 µM NaNO$_2$ pre-treatment the rate was 54 µM/hr. No differences were seen in the rate of nitrite production between each sample suggesting NarK2 is not a nitrate/nitrite antiporter.

To detect a possible interaction between NarK2 and nitrite, the export of nitrite was analyzed. Benzyl viologen in the reduced state is a nitrate and nitrite ionophore and can transport nitrate into the cell [24]. Nitrite production by the *M. tuberculosis* narK2 knockout mutant RVW3 was determined at pH 8.5 which would reduce the diffusion of both ions, and at the standard pH of 6.7. After incubation with nitrate and benzyl viologen the cells were separated from the medium and nitrite levels determined in both fractions (Figure 3). Without benzyl viologen or with the oxidized form there was no production of nitrite due to the defect in nitrate transport. When reduced benzyl viologen was added the nitrite...
levels in the medium increased. All nitrite was detected in the media, and none was associated with the cells indicating that the loss of narK2 did not affect the ability of M. tuberculosis to excrete nitrite.

For an independent approach NasA of Bacillus subtilis was expressed in M. tuberculosis. NasA is an assimilatory Type I nitrate transporter closely related to NarK2 of M. tuberculosis [22]. During assimilation nitrate is transported into B. subtilis where it is reduced to nitrite before being assimilated in the cytoplasm. Nitrite is not excreted before assimilation. nasA from B. subtilis under control of the M. tuberculosis narK2 promoter was integrated into the chromosome of M. tuberculosis in RVW3. As a control RVW3 expressing narK2 from the same plasmid was used. RVW3 complemented with narK2 showed low activity in aerobic cultures with strong induction in NRP-1 and NRP-2, in a manner very similar to wild type (Table 1). RVW3 expressing nasA produced similar levels of nitrite in NRP cultures indicating that NasA was able to complement the narK2 knockout.

RVW3 expressing either narK2 or the B. subtilis nasA from the integrated plasmid were grown in the Wayne model to induce the expression of both genes. The cells were centrifuged, resuspended in media at pH 8.5 and nitrate added. The cultures were incubated anaerobically for 3 hrs, the cells separated from the medium and nitrite levels determined. Similar levels of nitrite were detected in the cells of both strains; 0.7 μM ± 0.3 for RVW3 pNarK2, and 0.8 μM ± 0.3 for RVW3 pNasA indicating that export was not inhibited in the absence of narK2.

### Table 1. Nitrite levels in M. tuberculosis expressing B. subtilis nasA or M. tuberculosis narK2.

|          | AG* | NRP-1b | NRP-2c |
|----------|-----|--------|--------|
| RVW3 pNarK2 | 133±8 | 1013±35 | 2689±28 |
| RVW3 pNasA  | 258±6 | 925±14 | 2414±368 |

Mean nitrite concentration (micromolar) ± standard deviation.

*After 115 h of growth (final OD_{560} of ~0.4).

*After 115 h in the Wayne model.

*After 255 h in the Wayne model.

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**Mutagenesis of NarK2**

NarK2 of M. tuberculosis is a member of the major facilitator superfamily. Six computer models, DAS, HMMTOP, PREDTRM, SPLIT, TMHMM, and TMMPRED were used to identify possible membrane regions in the protein. A secondary structure was generated predicting 12 transmembrane spanning regions (TMS) (Figure 4). I-TASSER [25,26] was used to develop a 3D model consisting of two domains of the first six, and last six TMS regions.

A sequence comparison including both respiratory and assimilatory NNPs identified conserved amino acids agreeing with previous analysis [3,4,27]. Site-directed mutagenesis was used to change 11 of the conserved amino acids to serine, threonine or glycine. Little is known about the bacterial nitrate transporters that play a role in nitrate assimilation rather than respiration. To provide more information on this group, ten amino acids conserved in assimilatory but not respiratory NNPs were also selected for mutagenesis. Amino acids were changed to either serine or to the amino acid present in the assimilatory transporter. Finally, NarK, the nitrate transporter of E. coli contains 5 cysteines and it was proposed these might play a role in inactivating nitrate transport in the presence of oxygen [28]. These cysteines are not conserved in the M. tuberculosis NarK2 which does however contain 2 cysteines which could fulfill a redox sensing role. These two were changed to serines. A total of 23 mutations were created (Table 2). Each mutant gene was constructed in the previously characterized narK2 plasmid. The plasmids were then electroporated into the M. tuberculosis narK2 knockout strain RVW3, where they integrated into the chromosome. The presence of the mutated copies of narK2 were verified by Southern blot and DNA sequencing.

All of the point mutation produced no effect on aerobic growth or the shiftdown growth curves in the Wayne model (Data not shown). Nitrite production was measured during aerobic growth to verify that nitrate reductase enzyme levels were similar in the mutants (Table 2). All mutants including the knockout RVW3 showed similar levels of nitrite production during aerobic culture.

Wild type M. tuberculosis and the complemented RVW3 pNarK2 strain produced low levels of nitrite during aerobic growth, with high levels during both microaerobic (NRP-1) and anaerobic (NRP-2) conditions (Table 2). However, many mutants as well as RVW3 show a defect during hypoxia. The strongest effect was seen with P50S, R58S, G131S, Y215S and...
R259S which were effectively inactive. Mutants F19S, G69S, P84T, F114S, F140S, D267G and G315S showed different levels of inhibition. Some mutations, including those based on assimilatory transporters, had no effect on nitrite levels. All mutants with reduced activity in NRP-1 showed a similar defect in NRP-2.

**Effect of each Mutation on Redox Control**

To determine if each transporter was still inactivated by oxygen a culture of each mutant was grown into NRP-1. It was opened, nitrate added and then nitrite measured at intervals over 5 hrs (Figure 5). The rate of nitrite production by wild type NarK2 was approximately 15-fold higher during anaerobiosis in comparison to aerobic conditions. Each mutant was inactive suggesting no role for these amino acids in the redox control of NarK2.

**Redox Control of Nitrate Transport**

No mutants were identified that affected the inhibition of nitrite production by oxygen. Therefore the role of nitrate transport during oxygen inhibition was reexamined by analyzing the *M. tuberculosis* strain expressing the assimilatory *B. subtilis* NasA. Assimilatory nitrate transporters such as NasA function during aerobic growth and are not inactivated by the presence of oxygen. If this inactivation is due to a change in the proton gradient or other factor required for transport then NasA should also be inactivated when expressed in aerobic *M. tuberculosis*. If inactivation is due to NarK2 then NasA expressing cultures should show activity during aerobic incubation.

Nitrate production was measured in a NasA expressing strain under aerobic conditions. RVW3 strains expressing either *narK2* or *nasA* were grown into NRP-1. Nitrate was added and each culture was then incubated anaerobically (Figure 6). As seen previously (Table 1) RVW3 expressing *narK2* produced higher levels of nitrite than RVW3 with *nasA* during anaerobic incubation although both genes were under the control of the *narK2* promoter. If an NRP-1 culture expressing NarK2 was exposed to air a rapid decrease in nitrite production occurred. This decrease was not seen in cultures expressing NasA. This suggests that the nitrate transporter NarK2 is the point of control for oxygen inhibition. The *nasA* strain also showed slightly higher nitrite production during aerobic growth due to low expression of *nasA* from a plasmid promoter (Table 1).

**Discussion**

**Transport of Nitrate**

*M. tuberculosis* has the ability to persist for decades in humans despite cell-mediated immunity. The initial encounter of the host with *M. tuberculosis* results in phagocytosis of the bacteria by macrophages in the alveoli. This is followed by replication of the bacteria within unactivated macrophages. Organized granulomas are formed as delayed type hypersensitivity develops, and the number of bacteria plateaus. In humans these tubercle lesions are able to control bacterial replication and isolate the bacteria but not eliminate them completely.

Two stresses that *M. tuberculosis* is exposed to in granulomas are hypoxia and nitric oxide. Nitric oxide produced by macrophages breaks down by a variety of pathways with nitrate being one stable end product. *M. tuberculosis* responds to both hypoxia and nitric oxide by initiating a complex developmental program resulting in a non-replicating persistent state characteristic of latent infection [8,29]. The nitrate reductase system is induced to maintain the proton gradient for the continued production of ATP. This allows *M. tuberculosis* to survive the inhibition of respiration, and to provide energy during the development of the NRP state.

At physiological pH nitrate is an ion (pKₐ of HNO₃ = −1.3) which, along with the membrane potential of *M. tuberculosis* at −110 mV [30], limits diffusion into the cell. Porins allow nitrate to cross the first permeability barrier of the outer lipid layer of *M. tuberculosis* [31]. NarK2 then transports nitrate across the cell.
membrane to the cytoplasm where the active site of nitrate reductase is located.

In *Paracoccus pantotrophus* two nitrate transport systems were identified [32]. One, identified as a Type I nitrate/proton symporter utilized the proton gradient and was active when nitrate was initially added to the medium. As intracellular nitrate levels increased the second transporter, a Type II nitrate/nitrite symporter, and a Type II nitrate/nitrite symporter, was active. A nitrate/nitrite antiporter would seem to be required when nitrite is present in the medium. As intracellular nitrite levels increase, the process appears energetically unfavorable. The genome of *M. tuberculosis* encodes 3 additional homologues to narK2 named narK1, narK3 and *narU*. These 3 are predicted to be Type II nitrate/nitrite antiporters but none of them are induced during hypoxia [34,35]. In addition, in the Wayne model the narK2 knockout mutant showed only a low level of nitrate reductase activity attributed to diffusion suggesting NarK2 is the only transporter active under these conditions [13].

There are several factors that may explain why *M. tuberculosis* relies only on a H+/nitrate symporter. Although the reduction of nitrate in place of oxygen results in fewer protons exported [32] *M. tuberculosis* also responds to hypoxia *in vivo* and chronic infection in the mouse lung by switching from a proton-pumping to a non-proton-pumping NADH dehydrogenase [16]. Even in the presence of nitrate *M. tuberculosis* does not grow anaerobically, unlike *P. pantotrophus* and *E. coli* both of which use nitrate/nitrite antiporters during nitrate reduction. Without replication the energy demand of cells would greatly decrease. During anaerobiosis ATP synthesis and the proton motive force are still essential for survival [30,33] but additional functions of nitrate reductase may be more important during NRP in *M. tuberculosis*. These include redox balancing by NADH/NAD+ recycling, and the use of the proton-utilizing nitrate reductase reaction to reduce internal acid stress.

Nitrate can be toxic especially in combination with the acidic conditions of the macrophage. Since it is not reduced further *M. tuberculosis* must export the nitrate which may require additional energy. A nitrate transporter is required for maximum nitrate reductase activity suggesting the export of the nitrate would also require a nitrate exporter. A nitrate/nitrite antiporter would seem ideal for this purpose but NarK2 does not fulfill this role. The identity of this nitrate exporter is not known.

### Mutagenesis of narK2

The 3D structures of several members of the major facilitator superfamily have been determined and are all very similar [36–38]. Extensive mutagenic studies of some members have allowed detailed structural and functional analysis of these transporters but no detailed structure has been determined for an NNP member. In this study a detailed mutagenic analysis of the NarK2 protein of *M. tuberculosis* was performed which included sites that had not previously been mutated. It is possible to compare these results to previous mutagenic studies involving NNPs (Table 3) [27,39–43] and the 3D structure of related transporters.

A nitrate signature is present in all members of the NNP family including NarK2 [1,27,41,44]. In NarK2 it is found in the predicted intracellular loop preceding and including membrane spanning region 5 (amino acids 125–144) (Figure 2). Based on the similarity to the GlpT and LacY transporters this section, along with TSM 2, 8 and 11, make up the inner substrate binding region [36,38]. In NarK2 four residues in the signature were mutated, R129S, G131S, G140S and A145G. The mutation of glycine 131 produced a partially active protein as was seen with this residue in NrtA [27]. The loss of G140 produced a partially active protein as seen with this residue in NrtA [27]. The loss of the charged arginine at 129 had little effect suggesting the nitrate signature does not need this positive charge.

In the glyceral-3-phosphate transporter GlpT of *E. coli* two arginines bind the negatively charged phosphate of the substrate [38]. In NarK2 of *M. tuberculosis* two arginines (R58 and R259) were important for activity while a third, R129, was not. These two essential intramembrane arginines probably bind nitrate.

Treatment of *Paracoccus denitrificans* with phenylglyoxal reduced nitrate transport suggesting an important role for arginine in NarK [45]. In both NarK of *Paracoccus pantotrophus* [42] and NrtA of *A. nidulans* [27,41] the conserved arginines in TMS region 2 and 8 were identified as critical for activity. It was proposed the charged arginine directly interacts with nitrate [41]. Substitution of the

### Table 2. Nitrate reductase activity of narK2 mutants.

| Strain | Aerobic* | NRP-1b | NRP-2a |
|--------|----------|--------|--------|
| WT     | 115 ± 4  | 795 ± 43 | 2365 ± 61 |
| RW3    | 105 ± 5  | 24 ± 6  | 26 ± 3  |
| pNarK2 | 114 ± 4  | 1012 ± 36 | 2689 ± 28 |
| L8V    | 102 ± 5  | 710 ± 40 | 2192 ± 16 |
| F19S   | 102 ± 10 | 326 ± 26 | 1388 ± 173 |
| P50S   | 136 ± 15 | 118 ± 6  | 415 ± 27  |
| R58S   | 109 ± 4  | 21 ± 1   | 35 ± 1   |
| G69S   | 134 ± 10 | 620 ± 19 | 2083 ± 29 |
| G70P   | 118 ± 6  | 792 ± 49 | 2364 ± 54 |
| T78S   | 108 ± 3  | 830 ± 21 | 2318 ± 38 |
| P84T   | 111 ± 4  | 678 ± 21 | 1582 ± 59 |
| G89S   | 131 ± 6  | 835 ± 80 | 2621 ± 167 |
| A92G   | 108 ± 3  | 830 ± 47 | 2318 ± 38 |
| Y97S   | 122 ± 9  | 993 ± 84 | 2977 ± 23 |
| F114S  | 140 ± 3  | 464 ± 55 | 1340 ± 102 |
| I118L  | 126 ± 11 | 975 ± 100 | 2162 ± 76 |
| R129S  | 127 ± 6  | 950 ± 114 | 3179 ± 53 |
| G131S  | 104 ± 3  | 56 ± 8   | 254 ± 7  |
| G140S  | 125 ± 12 | 355 ± 35 | 1203 ± 68 |
| A145G  | 127 ± 5  | 794 ± 49 | 1951 ± 280 |
| Y215S  | 126 ± 6  | 39 ± 3   | 65 ± 5   |
| R259G  | 101 ± 17 | 26 ± 1   | 36 ± 9   |
| D267G  | 103 ± 3  | 337 ± 17 | 1258 ± 36 |
| C309S  | 111 ± 4  | 797 ± 35 | 2873 ± 208 |
| G315S  | 120 ± 4  | 454 ± 17 | 1364 ± 47 |
| C378S  | 108 ± 2  | 800 ± 39 | 2808 ± 132 |

Mean nitrite concentration (micromolar) ± standard deviation.

*After 115 h of growth (final OD600 of ~0.4).

*After 115 h in the Wayne model.

*After 255 h in the Wayne model.

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arginines with positively charged lysines reduced, but did not eliminate, activity in NrtA.

Mutation F19S resulted in reduced activity, and the mutation of residue L8V had no effect on activity. These residues are in a region (amino acids 10–20) similar to a motif conserved in nitrate transporters of eukaryotes. This region has been proposed to regulate the pore opening by closing it after the substrate binds [27].

Major facilitator superfamily members also contain two conserved regions between membrane spanning regions 2 and 3, as well as between 8 and 9 [1]. This region is proposed to maintain the curvature of TMS2 and TMS8 [38]. The mutation of 2 glycines to serines in the first region (G69S and G70P) had little effect on nitrite production suggesting the importance of the glycines may be their small size. A similar change in NarU of *E. coli* also had little effect [39]. A change in the conserved aspartic acid in the second signature (D267G) resulted in decreased activity in NarK2 of *M. tuberculosis* as well as NarU of *E. coli* [39](Table 3).

NarK2 has a high glycine content (39 out of 395 amino acids). In particular the proposed TMS region 5 has five glycines and TMS 11 has six (Figure 4). Four of the most highly conserved glycine residues were changed to serines, and three of these mutants (G131S, G140S, G315S) had reduced activity. This could indicate a requirement for tight packing in the membrane. Not every conserved amino acid was required as the G69S mutation showed no apparent change in activity.

The conserved proline at position 84 was important for activity in both the *M. tuberculosis* NarK2 and the *E. coli* NarU, but the conserved tyrosine at 215 was important only in NarK2 [39]. Proline, like glycine, is often important for flexibility and this could explain the importance of the intramembrane prolines at position 84 and 50.

It is possible that some of the mutants created here resulted in reduced expression of the mutant NarK2. However in other mutational studies with nitrate transporters, no effect on expression was seen [27,39–41]. Some of these mutations may affect insertion of NarK2 into the membrane although computer analysis did not identify any obvious changes in structure. However without an antibody to NarK2 it is not possible to determine protein levels or location at this time.

Major facilitator superfamily members also contain two conserved regions between membrane spanning regions 2 and 3, as well as between 8 and 9 [1]. This region is proposed to maintain the curvature of TMS2 and TMS8 [38]. The mutation of 2 glycines to serines in the first region (G69S and G70P) had little effect on nitrite production suggesting the importance of the glycines may be their small size. A similar change in NarU of *E. coli* also had little effect [39]. A change in the conserved aspartic acid in the second signature (D267G) resulted in decreased activity in NarK2 of *M. tuberculosis* as well as NarU of *E. coli* [39](Table 3).

NarK2 has a high glycine content (39 out of 395 amino acids). In particular the proposed TMS region 5 has five glycines and TMS 11 has six (Figure 4). Four of the most highly conserved glycine residues were changed to serines, and three of these mutants (G131S, G140S, G315S) had reduced activity. This could indicate a requirement for tight packing in the membrane. Not every conserved amino acid was required as the G69S mutation showed no apparent change in activity.

The conserved proline at position 84 was important for activity in both the *M. tuberculosis* NarK2 and the *E. coli* NarU, but the conserved tyrosine at 215 was important only in NarK2 [39]. Proline, like glycine, is often important for flexibility and this could explain the importance of the intramembrane prolines at position 84 and 50.

It is possible that some of the mutants created here resulted in reduced expression of the mutant NarK2. However in other mutational studies with nitrate transporters, no effect on expression was seen [27,39–41]. Some of these mutations may affect insertion of NarK2 into the membrane although computer analysis did not identify any obvious changes in structure. However without an antibody to NarK2 it is not possible to determine protein levels or location at this time.

Figure 5. Oxygen inactivation of NarK2 mutants. The activity of each mutant was measured following exposure to oxygen. Wild type *M. tuberculosis* cultures incubated either anaerobically (WT-AN) or aerobically (WT) were included as controls. The standard deviation is indicated. doi:10.1371/journal.pone.0045459.g005

Figure 6. Oxygen inactivation of NarK2 but not NasA. Nitrite levels were determined in cultures of RVW3 pNarK2 (circles) and RVW3 pNasA (triangles). NRP-1 cultures were incubated anaerobically for 8 h (solid symbols), or anaerobically for 4 hrs, opened and then incubated aerobically for an additional 4 h (empty symbols). The arrow indicates when the cultures were shifted from anaerobic to aerobic conditions. The standard deviation is indicated. doi:10.1371/journal.pone.0045459.g006
Table 3. Comparison of the Effect of Mutations in NarK2 to Mutations in the Comparable Amino Acids in Other Nitrate/Nitrite Porters.

| Strain | NarK2 | NrtA\* | NarU\* | NarK\* |
|--------|-------|--------|--------|--------|
| LBV    | No effect |        |        |        |
| F195   | Inhibit | F47-Inhibit |      |        |
| P505   | Inhibit |        |        |        |
| R58S   | Inactive | R87-inhibit/Inactive | R66-Inactive | R66/R520-Inactive |
| G69S   | Inhibit |        |        |        |
| G70P   | No effect | G99-No effect |      |        |
| T78S   | No effect | P113-No effect/Inhibit |      |        |
| P84T   | Inhibit |        |        |        |
| G89S   | No effect |        |        |        |
| A92G   | No effect |        |        |        |
| Y97S   | No effect |        |        |        |
| F114S  | Inhibit | F145-Inactive |      |        |
| I118L  | No effect |        |        |        |
| R129S  | No effect |        |        |        |
| G131S  | Inactive | G157-Inactive | G162-inactive |        |
| G140S  | Inhibit | G167-Inhibit | G172-No effect/Inactive |        |
| A145G  | No effect | G172-Inhibit |      |        |
| Y215S  | Inactive | Y261-No effect |      |        |
| R259G  | Inactive | R368-Inhibited/Inactive | R269/R736-Inactive |        |
| D267G  | Inhibit | D311-Inactive |      |        |
| C309S  | No effect |        |        |        |
| G315S  | Inhibit | G433-Inhibit |      |        |
| C378S  | No effect |        |        |        |

Redox Effect

Nitrate reduction is regulated by control of nitrate transport in *M. tuberculosis*. Transcription of *narK2* is induced by hypoxia, while the activity of this transporter is controlled by redox levels in the cell [15,17]. Even in the presence of oxygen, NarK2 remains active if respiration is inhibited, for example by nitric oxide [17]. Nitrate transporters may sense the redox state of the cell by interacting with the quinone pool [17,21]. Quinone interacts with proteins at Q Sites which have only weak similarity making them difficult to predict [46,47]. There are no obvious Q sites in the *M. tuberculosis* NarK2 (Data not show).

Transport of nitrate in *E. coli* was sensitive to N-ethylmaleimide which could indicate a role for redox sensitive sulfhydryl groups [48]. When the *E. coli* narK was sequenced the five cysteines that were encoded were proposed to play a role in redox control [28]. Mutating the two cysteines of NarK2 to serines resulted in no change in activity indicating they are not essential for function. In the assimilatory nitrate transporter of *Aspergillus nidulans*, NrtA none of the eight cysteines were essential [40]. In NarK2 both cysteine mutants were inactive in the presence of oxygen suggesting no role in redox inactivation (Figure 5).

None of the mutants showed significant activity in the presence of oxygen. NrtA however was active showing that NarK2 is the point of redox inactivation (Figure 6). The reason for the dual hypoxic control of *narK2*, transcription and activity, is unknown.

There was no noticeable defect in the strain expressing the oxygen insensitive NrtA. Growth, shiftdown and recovery from hypoxia were all similar to the *narK2* expressing strain (Data not shown).

Supporting Information

Table S1 Primers used in this study (DOC)

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Author Contributions

Conceived and designed the experiments: CDS RWR. Performed the experiments: MG MM RWR CDS. Analyzed the data: MG CDS. Contributed reagents/materials/analysis tools: RWR CDS. Wrote the paper: CDS.

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