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Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in *Salmonella enterica* serovar Typhimurium

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The production of cytotoxic nitric oxide (NO) and conversion into the neuropharmacological agent and potent greenhouse gas nitrous oxide (N_2O) is linked with anoxic nitrate catabolism by *Salmonella enterica* serovar Typhimurium. *Salmonella* can synthesize two types of nitrate reductase: a membrane-bound form (Nar) and a periplasmic form (Nap). Nitrate catabolism was studied under nitrate-rich and nitrate-limited conditions in chemostat cultures following transition from oxic to anoxic conditions. Intracellular NO production was reported qualitatively by assessing transcription of the NO-regulated genes encoding flavohaemoglobin (Hmp), flavorubredoxin (NorV) and hybrid cluster protein (Hcp). A more quantitative analysis of the extent of NO formation was gained by measuring production of N_2O, the end-product of anoxic NO-detoxification. Under nitrate-rich conditions, the *nar*, *nap*, *hmp*, *norV* and *hcp* genes were all induced following transition from the oxic to anoxic state, and 20 % of nitrate consumed in steady-state was released as N_2O when nitrite had accumulated to millimolar levels. The kinetics of nitrate consumption, nitrite accumulation and N_2O production were similar to those of wild-type in nitrate-sufficient cultures of a *nap* mutant. In contrast, in a *narG* mutant, the steady-state rate of N_2O production was ~30-fold lower than that of the wild-type. Under nitrate-limited conditions, *nap*, but not *nar*, was up-regulated following transition from oxic to anoxic metabolism and very little N_2O production was observed. Thus a combination of nitrate-sufficiency, nitrite accumulation and an active Nar-type nitrate reductase leads to NO and thence N_2O production, and this can account for up to 20 % of the nitrate catabolized.

Key words: Enterobacteriaceae, nitrate reductase, nitric oxide, nitrite reductase, nitrous oxide, *Salmonella*.

INTRODUCTION

The Enterobacteriaceae family of Gammaproteobacteria are found naturally in soils, water systems and sewage and as a part of the gut flora in the gastrointestinal tract. They are facultative anaerobes that can, with few exceptions, use nitrate or nitrite as terminal respiratory electron acceptors. The availability of these electron acceptors varies in the different environments to which the bacteria adapt [1]. For an enteric pathogen, for example, the gastrointestinal tract can be rich in nitrate or nitrite, but nitrate is more scarce in bodily fluids such as the bloodstream [1]. In many species of Enterobacteriaceae, there are two biochemically distinct nitrate reductases: one membrane-bound with the active site located in the cytoplasm and the other in the periplasm. These are coupled to two nitrite reductases to provide parallel pathways for nitrate reduction to ammonium in the two cellular compartments and that are differentially expressed in response to different nitrate and nitrite concentrations [1,2]. In the cytoplasm, nitrate is reduced to nitrite by a membrane-bound respiratory nitrate reductase system (NarGHI):

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \text{ (Reaction 1).}$$

The nitrite produced can then be reduced further to ammonium by a sirohaem-containing nitrite reductase (NirB):

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \text{ (Reaction 2).}$$

In the periplasm, the process involves two different enzymes: a periplasmic nitrate reductase (NapA) that reduces nitrate to nitrite and a periplasmic cytochrome *c* nitrite reductase (NrfA) that further reduces the nitrite to ammonium. Reactions 1 and 2 together lead to the production of extracellular ammonium and are often termed DNRA (dissimilatory nitrate reduction to ammonium).

Salmonella and *Escherichia coli* produce the cytotoxin nitric oxide (NO) as a side-product of nitrate or nitrite metabolism [3,4]. One major source of this NO has been suggested to be the reduction of nitrite by the membrane-bound nitrate reductase NarG [3]. This endogenous NO leads to derepression of genes encoding systems that are concerned with the detoxification of NO and the repair of proteins damaged by the cytotoxin. The regulator that mediates this derepression is the NO-binding protein NsrR (NO-sensing repressor) [5–7]. A key enzyme in the NsrR regulon in *Salmonella enterica* serovar Typhimurium is flavohaemoglobin (Hmp) that reduces two molecules of NO to one molecule of nitrous oxide (N_2O) under anoxic conditions, using cytoplasmic NADH as electron donor [8,9]. This represents the conversion of a potent cytotoxin into a product that is both a neuropharmacological agent and potent greenhouse gas [10]. There have been reports of N_2O release by pure cultures

Abbreviations used: D, dilution rate; DNRA, dissimilatory nitrate reduction to ammonium; Hmp, flavohaemoglobin; Km^R, kanamycin-resistance; MS, minimal salts; NapA, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase; NirB, cytoplasmic sirohaem nitrite reductase; NorV, flavorubredoxin; NrfA, periplasmic cytochrome *c* nitrite reductase; NsrR, NO-sensing repressor; qc, specific rate of consumption; qRT, quantitative real-time; S_{BR}, bioreactor concentration of substrate; S_R, reservoir concentration.

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Table 1 All strains and plasmids used in the present study

Strain or plasmid	Genotypes or relevant characteristics	Reference
Strains		
SL1344	*Salmonella* Typhimurium, *his*$^-$, mouse-virulent	[40]
Δ*hmpA*	SL1344 Δ*hmpA*::kan	[29]
Δ*norV*	SL1344 Δ*norV*::cat	[29]
Δ*norV*Δ*hmpA*	SL1344 Δ*hmpA*::kan Δ*norV*::cat	[29]
Δ*narGHJI*	SL1344 Δ*narGHJI*::kan	The present study
Δ*napFDAGHBC*	SL1344 Δ*napFDAGHBC*::kan	The present study
Δ*nirB*	SL1344 Δ*nirB*::kan	The present study
Δ*hcp*	SI1344 Δ*hcp*::kan	The present study
Plasmids		
pKD4	Ap^R, pANT-Sγ derivative containing an FRT-flanked Km^R	[17]
pKD46	Ap^R, pINT-ts derivative containing *araC*-P$_{araB}$ and γ, β, *exo* genes	[17]

of Enterobacteriaceae, including *Escherichia coli*, *Klebsiella pneumoniae* and *S. enterica* during nitrate metabolism, and it is likely that this is due to reductive detoxification of NO produced as a side-product of nitrate metabolism [11,12,18–20]. In the present paper, we describe a biochemical study on nitrate catabolism in continuous cultures of the food-borne pathogen *Salmonella* Typhimurium which reveals that intracellular NO production and associated extracellular N_2O production can account for up to 20 % of nitrate catabolized and is linked to the culture nitrate status, nitrite accumulation from nitrate respiration and the biochemical type of the nitrate reductase system expressed.

EXPERIMENTAL

Bacterial strains and growth media

Salmonella Typhimurium strains (Table 1) were cultivated anaerobically in MS (minimal salts) medium [13]. The sole carbon and electron source was glycerol, the sole terminal electron acceptor was sodium nitrate, with ammonium (15 mM) present as a nitrogen source. Continuous culture was performed in a New Brunswick Scientific BioFlo 3000 fermenter with a 1.2 litre working volume under pH control (pH 7.0, 1 M NaOH and 1 M HCl/0.1 M H_2SO_4 used for regulation). A 100 ml volume of MS medium was inoculated with 5 ml of an overnight culture and aerobically incubated overnight at 37 °C. Then, 50 ml of this culture was used to inoculate the bioreactor. After 24 h of aerobic batch growth, the air supply was switched off and a feed of MS medium was started to achieve a dilution rate (D) of 0.04 h^{-1}. The measured dissolved O_2 (percentage air saturation) in the culture fell from 100 % to 0 % within 1 h of switching off the air supply and was monitored throughout the continuous culture phase of the experiment to ensure that it remained at 0 %. During the experiment, samples were taken at regular intervals to determine the attenuance at 600 nm (D_{600}), protein concentration and nitrogen compound composition.

Protein and nitrogen compound quantification

Protein concentration was determined using the method described in [14]. Nitrate was determined via HPLC using the anion-exchange column Ion Pac AS22, 2 mm×250 mm (Dionex, ICS-900) as described by the manufacturer. Nitrite was measured colorimetrically with a modified Griess reaction [15]. A PerkinElmer Clarus® 500 Gas Chromatograph with an ECD (Electron Capture Detector) and Elite-PLOT Q (DVB Plot Column, 30 m length; 0.53 mm internal diameter; carrier gas, N_2; make-up gas, 95 % argon/5 % methane; temperatures as described by the manufacturer) were used with known gas standards of N_2O [0.4, 100, 1000 p.p.m., supplied from StGas] to determine the N_2O concentration in headspace gas samples. From this, the total N_2O in headspace and solution was calculated by applying Henry's Law, assuming equilibration between the solution and gas phases and using a Henry's Law constant at 37 °C of 0.453.

Enzyme activity

The NarGH nitrate reductase complex, comprising the 140000 Da NarG and the 60000 Da NarH subunits, was purified from anaerobic cultures of *Salmonella* Typhimurium and assayed using Methyl Viologen as the electron donor, essentially as described previously for *Paracoccus pantotrophus* NarGH [16].

RNA extraction, qRT (quantitative real-time)-PCR and mutant construction

RNA was extracted at the appropriate time points from nitrate-sufficient and -limited continuous cultures of *Salmonella* Typhimurium SL1344 using a Promega SV 96 total RNA purification kit. The total RNAs were first treated with Turbo DNaseFree from Ambion and the absence of DNA contamination was verified by PCR. RNA quality was assessed on an Agilent 2100 Bioanalyser. Then, 2 μg of DNaseI-treated total RNA were retro-transcribed from random hexamers (Invitrogen) with Superscript II RT (Invitrogen) according to the manufacturer's recommendations. Specific primers for the genes of interest amplifying an average product of 100 bp with an approximate T_m (melting temperature) of 60 °C were designed. The qRT-PCRs were performed on a 5-fold dilution of the total cDNA obtained, using the Bio-Rad Laboratories CFX96 instrument and SensiMix™ SYBR No-ROX kit (Bioline). The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* gene, expression of which is invariant across a large range of growth conditions. Mutants were constructed in *Salmonella* Typhimurium SL1344 using λ Red mutagenesis [17]. Oligonucleotides were designed which deleted the entire gene or operon in question inclusive of start and stop codons. A linear PCR product was generated using a template plasmid, pKD4, resulting in a Km^R (kanamycin-resistance) gene cassette with 40 bp of homologous sequence flanking to the loci. The amplified DNA fragment was column-purified and electroporated into *Salmonella* Typhimurium SL1344pKD46. Mutations were confirmed by PCR using primers external to the site of mutagenesis; as well as internal

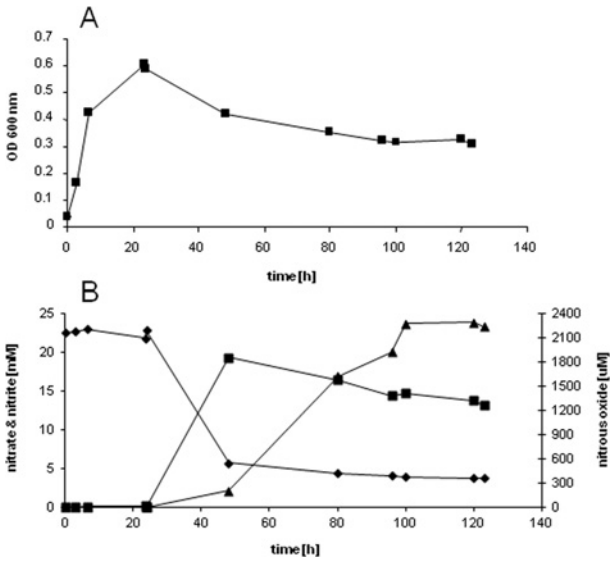

Figure 1 Nitrate, nitrite and N_2O consumption or production in a glycerol-limited nitrate-sufficient continuous culture of *Salmonella* Typhimurium SL1344

The culture was initially grown in batch mode under atmospheric oxygen concentration for 24 h, during which time cellular biomass [indicated by an increase in D_{600} ('OD')] was generated. The air supply to the culture was then switched off and the system switched to continuous mode at a dilution rate of 0.04 h^{-1}. The measured dissolved O_2 (percentage air saturation) in the culture fell from 100 % to 0 % within 1 h of switching off the air supply, and was monitored throughout the experiment to ensure it remained at 0 %. The pH and temperature were maintained at 7 and 37 °C respectively. The glycerol concentration in the reservoir feed was 5 mM and the nitrate concentration was 22.5 mM. (**A**) Biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). For clarity, only the results from a single chemostat run are shown. The results (± S.D.) derived from replicate experiments is given in Table 2.

primers within the Km^R cassette, k1 and k2 [17]. P22 transduction was used to transfer the mutations into a clean SL1344 background.

RESULTS

Nitrate respiration and N_2O production in nitrate-sufficient continuous cultures

Salmonella Typhimurium was cultured to anoxic steady-state in continuous cultures with nitrate (reservoir concentration, S_R, 22.5 mM) present as the respiratory electron acceptor and glycerol (S_R 5 mM) present as the carbon source for anabolism and electron source for respiration. The cultures were grown in batch mode under an atmospheric oxygen concentration for 24 h, during which time cellular biomass (x) was generated (Figure 1A). The air supply to the culture was then switched off and the system was switched to continuous mode ($D = 0.04\ h^{-1}$). There was a decrease in D_{600} and protein concentration in the bioreactor as the culture shifted from aerobic to anaerobic metabolism, until a new biomass steady-state was reached after three or four bioreactor vessel volume changes (~80–120 h). During the transitional, non-steady-state, phase (between 24 and ~80 h) the nitrate concentration in the bioreactor ($S_{BR}NO_3^-$) decreased, consistent with a shift from oxygen respiration to anaerobic nitrate respiration (Figure 1B). The shift to nitrate respiration was also reflected by increased transcription of both the *narG* and *napA* nitrate reductase genes and the gene for the nitrate/nitrite antiporter *narK*, detected using qRT-PCR analysis, at 80 h and 120 h compared with 5 h (Figure 2).

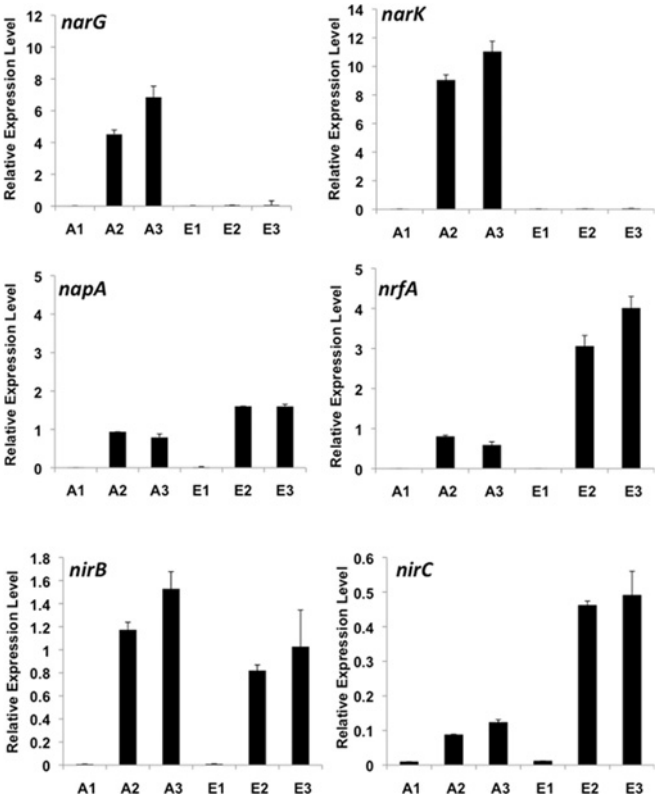

Figure 2 qRT-PCR of genes involved in nitrate and nitrite transport and reduction

The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* control. Results are means ± S.D. A, nitrate sufficiency; E, nitrate limitation; 1, 5 h oxic; 2, 80 h anoxic; 3, 120 h anoxic.

Nitrite was produced almost stoichiometrically with nitrate consumption during the first ~20 h of the transition phase (24–48 h) (Figure 1B). This is consistent with nitrite being produced directly from nitrate reduction by the nitrate reductase(s) (Reaction 1), such that the $S_{BR}NO_3^-$ decreased from ~22 mM to ~5 mM and the $S_{BR}NO_2^-$ increased from ~0 mM to ~18 mM (Figure 1B). Over the next 50 h, the $S_{BR}NO_2^-$ decreased by ~4.5 mM from ~18 mM to a steady-state value of ~14 mM (100–120 h). In steady state, the specific rate of nitrate consumption ($qcNO_3^-$) was ~20 % higher than the specific rate of nitrite accumulation ($qpNO_2^-$) (Table 2). To account for this difference some of the nitrite produced from nitrate in Reaction 1 must be further consumed by the culture. This rate of consumption ($qcNO_2^-$) can be estimated from the difference between $qcNO_3^-$ and $qpNO_2^-$ (Table 2). Ammonium is a possible net product of nitrite reduction by the NirB or NrfA nitrite reductases (Reaction 2), with *nirB* in particular being strongly induced during the anaerobic phase (Figure 2). However, the $S_{BR}NH_4^+$ remained constant at ~13 mM throughout the continuous culture phase of the experiment, which represented a net consumption of ~2 mM of the 15 mM NH_4^+ in the reservoir feed for anabolic purposes.

NO is also a potential net product of nitrite reduction, but extracellular accumulation was not detected above 1 μM. However, increased transcription of *hmp*, *hcp* and *norV* was observed following the transition from oxic (5 h sampling time) to anoxic (80 and 100 h sampling times) conditions (Figure 3). These genes are regulated by the cytoplasmic NO-responsive transcription factors NsrR (*hmp* and *hcp*) and NorR (*norV*),

Table 2 The steady-state rates of nitrate, nitrite and N_2O production in chemostat cultures of *Salmonella* Typhimurium

The data presented are taken during steady state at 100–120 h from replicate continuous cultures run in parallel under identical conditions. $qcNO_3^- = (S_RNO_3^- - S_{BR}NO_3^-)D/x$; $qpNO_2^- = (S_{BR}NO_2^-)D/x$; $qcNO_2^- = qcNO_3^- - qpNO_2^-$; $qpN_2O = (S_{BR}N_2O)D/x$. N_2O data are expressed as nitrogen-equivalents to allow for direct comparison between N_2O and nitrate or nitrite. ND, not detectable, nitrite detection limit = 0.005 mM.

Strain	NO_3^-	Glycerol	Biomass ($g \cdot l^{-1}$)	NO_3^- consumed ($mmol \cdot l^{-1}$)	NO_2^- produced ($mmol \cdot l^{-1}$)	N_2O produced ($mmol \cdot l^{-1}$)	$qcNO_3^-$ ($mmol \cdot g^{-1} \cdot h^{-1}$)	$qpNO_2^-$ ($mmol \cdot g^{-1} \cdot h^{-1}$)	$qcNO_2^-$	qpN_2O ($mmol \cdot g^{-1} \cdot h^{-1}$)[1)]
SL1344	22.5	5	0.19 ± 0.01	18.1 ± 0.7	14.0 ± 0.3	4.0 ± 0.2	3.81 ± 0.19	2.95 ± 0.15	0.86	0.850 ± 0.050
SL1344	5.5	22	0.18 ± 0.01	5.0 ± 0.5	ND	0.016 ± 0.003	1.11 ± 0.10	ND	1.11	0.004 ± 0.001
nar	22.5	5	0.11 ± 0.01	16.0 ± 0.5	16.5 ± 1.0	0.076 ± 0.004	6.0 ± 0.6	6.0 ± 0.6	0	0.028 ± 0.003
nap	22.5	5	0.15 ± 0.02	21.0 ± 0.2	15 ± 1.0	3.6 ± 0.4	5.6 ± 1.0	4.0 ± 0.5	1.60	0.96 ± 0.10

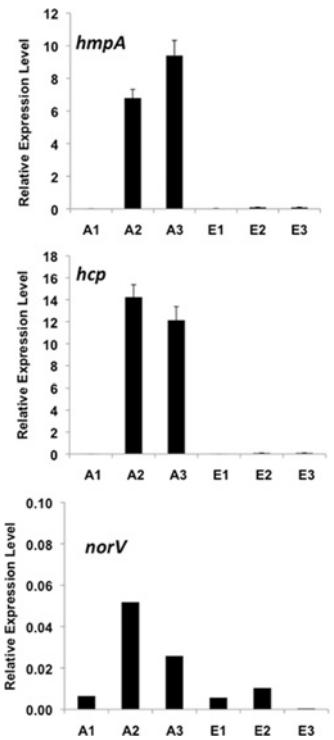

Figure 3 qRT-PCR of genes regulated by the NO-responsive transcription factors NsrR and NorR

The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* control. Results are means ± S.D. A, nitrate sufficiency; E, nitrate limitation; 1, 5 h oxic; 2, 80 h anoxic; 3, 120 h anoxic.

and so this increased transcription is indicative of intracellular NO production. It was notable that N_2O accumulated as the $S_{BR}NO_2^-$ decreased in the transition phase between 48 and 100 h (Figure 1B). The steady-state rate of N_2O production (qpN_2O), when normalized for the two nitrogens in N_2O compared with the one nitrogen in NO_2^-, matched the steady-state rate of nitrite consumption (Table 2), showing that this N_2O production is closely linked to the metabolism of nitrite generated from nitrate metabolism. Since *hmp*, *hcp* and *norV* transcription indicated the production of intracellular NO, then the series of reactions that lead to 2 mol of NO_2^- being reduced to 1 mol of N_2O via NO is predicted to be:

$$2[NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O] \text{ (Reaction 3)}$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \text{ (Reaction 4)}$$

Reaction 4 is known to be catalysed by both the Hmp and NorV (flavorubredoxin) enzymes under anoxic conditions, and so *hmp* and *norV* mutants were also analysed under the nitrate-sufficient continuous culture conditions. In the case of strains carrying single lesions in either *hmp* or *hcp*, the rate of N_2O production in steady state was comparable with wild-type. However, a double *hmp nor* mutant only produced N_2O at ~40 % of the rate of the wild-type, suggesting functional overlap of these two systems in NO detoxification and N_2O production under anoxic conditions. In total, ~18 mM nitrate in the feed reservoir was consumed in the steady state ($S_RNO_3^- - S_{BR}NO_3^-$ at $t = 120$ h) and ~4 mM nitrogen equivalents of N_2O was produced (Table 2). This represents a conversion of ~20 % of nitrate into N_2O (Table 2).

Comparison of N_2O production in *Salmonella* Typhimurium *nar* and *nap* mutants in nitrate-sufficient continuous cultures

The NarG nitrate reductase has previously been implicated in both NO and N_2O production and nitrosation in Enterobacteriaceae [2,3,11,12,18–20]. However, a number of Enterobacteriaceae species only have a Nap type of nitrate reductase [2], and, in those that have both Nar and Nap, the nitrate-rich growth conditions under which many previous studies of NO or N_2O production has been made would not be those that favour *nap* expression, which is maximal under nitrate-limiting conditions [1,2,5,21–23]. Thus the question of whether activity of Nap can lead to NO and N_2O production has not been directly addressed before. To investigate this, isogenic *narG* and *napA* strains were constructed. Both were able to grow under anaerobic conditions with nitrate as a sole electron acceptor, but a double *narG napA* mutant could not. Under the nitrate-rich continuous culture conditions, the *nar* strain, which is dependent on Nap for growth, achieved a steady-state anoxic biomass of ~60 % of the wild-type strain under identical culture conditions (Figure 4A and Table 2). The kinetics of nitrate consumption and nitrite accumulation during the aerobic–anaerobic transition phase were closely matched (Figure 4B and Table 2), suggesting that the respiratory nitrite reductase systems Nrf or Nir (Reaction 2) do not operate at a significant level to consume the nitrite produced from Reaction 1. The rate of nitrate consumption in the steady state (100–120 h) was comparable with that of the wild-type (Table 2). Significantly, however, the steady-state $S_{BR}N_2O$ was only ~0.08 mM in the *nar* strain (Figure 4B and Table 2), such that the steady-state rate of N_2O production (qpN_2O) was approximately 30-fold lower than for the wild-type strain (Table 2). In contrast, the rate of N_2O production in the *nap* strain was comparable with that of

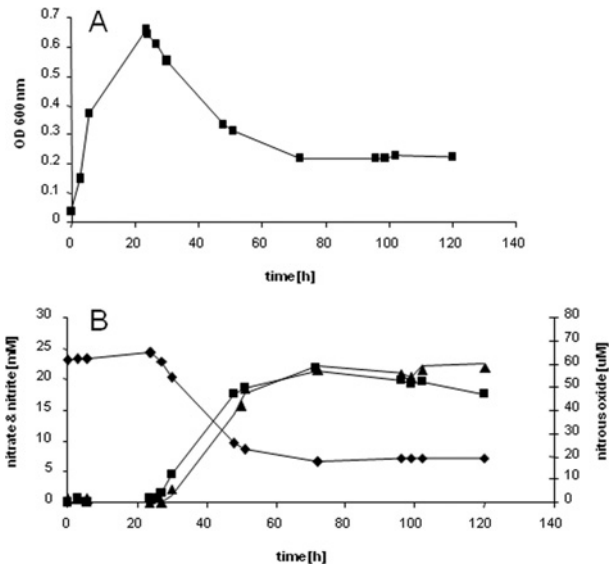

Figure 4 Nitrate, nitrite and N_2O consumption or production in a glycerol-limited nitrate-sufficient continuous culture of an *Salmonella* Typhimurium mutant deficient in Nar

The culture was run as described for Figure 1. The glycerol concentration in the reservoir feed was 5 mM and nitrate concentration was 22 mM. (**A**) Biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). For clarity, only the results from a single chemostat run are shown. The results ($\pm$S.D.) derived from replicate experiments is given in Table 2.

the wild-type (Table 2). The large difference in the rate of N_2O production between the *nap* and *nar* strains (Table 2) suggests that it is not associated with respiration in nitrate-rich cultures conditions itself, but with metabolism via the Nar system, rather than the Nap system, under nitrate-rich culture conditions.

NarG generates nitrite in the cytoplasm and it has been argued, on the basis of genetic and microbiological data, that this can compete with nitrate for the Nar active site in the cytoplasm [3], with Nar then catalysing Reaction 3. To demonstrate biochemically that purified *Salmonella* Nar can reduce nitrite, we isolated the membrane-associated NarGH complex from detergent-solubilized membranes of *Salmonella* Typhimurium. The enzyme complex displayed nitrate and nitrite reductase activities that each obeyed a Michaelis–Menten-type dependency on substrate concentration (Figure 5). These activities were fully sensitive to low concentrations of azide (20 μM), which is a potent inhibitor of Nar-type nitrate reductases and NO production and nitrosation by Enterobacteriacaea [19,24]. The K_m and V_{max} values for nitrate reduction were determined as 123 ± 14 μM and 83 ± 10 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively (Figure 5A). For nitrite reduction, the K_m and V_{max} values were determined as 5200 ± 1900 μM and 24 ± 5 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively (Figure 5B). Using a molecular mass for NarGH of 200 000 Da, the k_{cat} values for nitrate and nitrite could be approximated as 270 s^{-1} and 80 s^{-1} respectively.

In addition to Nar reducing nitrite to NO, it is possible that nitrite generated in the cytoplasm by Nar is a substrate for another NO-generating nitrite-reducing enzyme. The likely candidate for this would be the cytoplasmic NADH-dependent nitrite reductase NirB, which was also induced following transition to anoxic steady state (Figure 2), and has been implicated previously in cytoplasmic NO generation [25]. A *nirB* strain was therefore constructed and grown in the nitrate-rich continuous cultures, but the behaviour was identical with that of wild-type, with the specific rate of N_2O production was similar to that of wild-type.

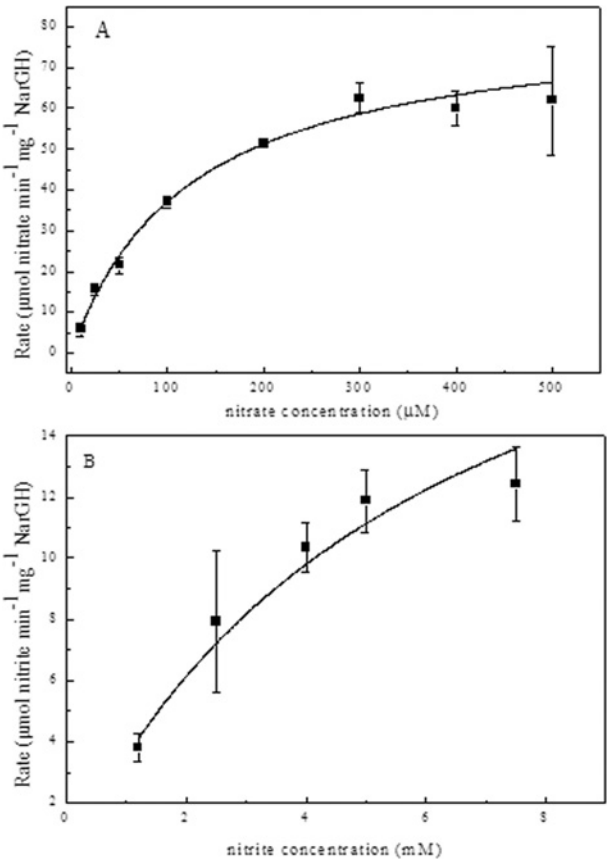

Figure 5 The kinetics of nitrate (A) nitrite (B) reduction by NarGH from *Salmonella* Typhimurium

Assays were undertaken in 20 mM Hepes and 2 mM EDTA (pH 7.0) under anaerobic conditions in nitrogen-sparged sealed cuvettes using reduced Methyl Viologen as electron donor. The data are fitted to the Michaelis–Menten kinetic model with the K_m and V_{max} values for nitrate reduction as 123 ± 14 μM and 82 ± 10 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively, and K_m and V_{max} values for nitrite reduction as 5200 ± 1900 μM and 24 ± 5 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively.

The NrfA nitrite reductase system has been shown previously to play a role in detoxifying NO added exogenously to *Salmonella* and *E. coli* [26–30]. Some reports have suggested that a product of NO metabolism by Nrf can be N_2O [31]. To assess this, a *nrfA* periplasmic nitrite reductase mutant was also examined in nitrate-rich continuous culture, but, like the *nirB* mutant, it behaved identically with wild-type. Thus, although *nrfA* was expressed in the anoxic phase of the cultures (Figure 2), the periplasmic NrfA enzyme does not appear to be important for detoxification of endogenously produced intracellular NO.

Nitrate metabolism in nitrate-limited continuous cultures

The net consumption of $\sim$17 mM nitrate and 5 mM glycerol by the nitrate-sufficient cultures suggested that running the continuous cultures with a $S_R NO_3^-$ of $<$17 mM and a $S_R$glycerol of $>$5 mM would lead to nitrate limitation. To achieve this condition, the $S_R NO_3^-$ was lowered to 5.5 mM and the $S_R$glycerol concentration increased to 22 mM (Figure 6A). Under these conditions, the $S_{BR} NO_3^-$ was very low under steady-state conditions (100–120 h) (Figure 6B), consistent with nitrate limitation. qRT-PCR revealed up-regulation of *nap*, but not *narG*, following the oxic–anoxic transition, consistent with Nap being the enzyme of choice for metabolism under nitrate-limited

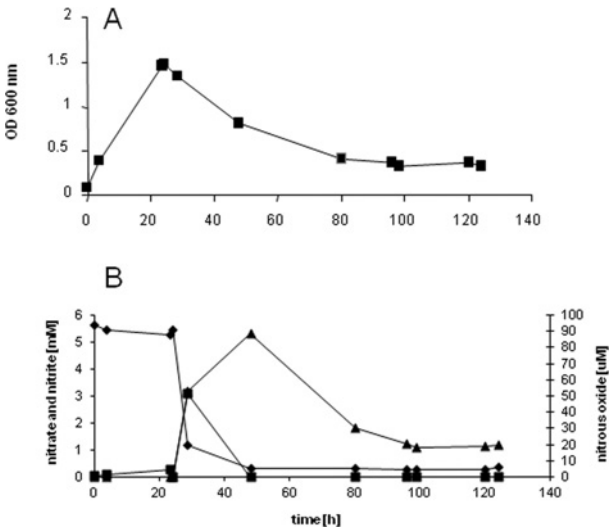

Figure 6 Nitrate, nitrite and N_2O consumption or production in a glycerol-sufficient nitrate-limited continuous culture of *Salmonella* Typhimurium SL1344

The culture was run as described for Figure 1. The glycerol concentration in the reservoir feed was 22 mM and nitrate concentration was 5 mM. (**A**) biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). Results are illustrative for experiments run in triplicate. For clarity, only the results from a single chemostat run are shown. The results ($\pm$ S.D.) derived from replicate experiments are given in Table 2.

conditions (Figure 2). During the transition phase of 24–48 h, nitrite accumulated in the reactor vessel to a maximum of $\sim$3 mM, concomitantly with nitrate consumption. However, this was only transient, and nitrite was not detectable (detection limit of 0.005 mM) when the cultures reached steady state (Figure 6B). Thus the rates of nitrate and nitrite consumption (Reactions 1 and 2) were matched (Table 2). Significantly, the profile of N_2O accumulation was quite different from the nitrate-sufficient cultures. Like nitrite, N_2O accumulated transiently, and the peak of production lagged approximately 20 h behind that of nitrite, but the maximum obtained was 20-fold lower than the maximum obtained in the nitrate-sufficient cultures (compare Figure 6B with Figure 1B). In steady state (100–120 h), there was only minimal N_2O release, the rate of which was more than two orders of magnitude lower than for the nitrate-sufficient cultures (Figure 6B and Table 2), with less than 0.1 % of the nitrate-nitrogen ending up as N_2O.

DISCUSSION

In the present study, we have examined nitrate catabolism and associated exogenous N_2O production during continuous culture of *Salmonella* Typhimurium under nitrate-rich and nitrate-limited anoxic conditions. NO is detoxified by conversion into N_2O in the cytoplasm (Reaction 4), and, as a consequence, the direct measurement of NO released by bacteria will grossly underestimate the actual level produced intracellularly during nitrate metabolism. *Salmonella* Typhimurium cannot reduce N_2O and so measuring its extracellular release is a good quantitative measure for the fraction of nitrate catabolized that forms NO intracellularly. This has been illustrated in the present study where, under nitrate-rich conditions, millimolar levels of extracellular N_2O were measured, but extracellular NO was not detected above 1 μM. This demonstrates that, under the steady-state metabolic conditions established, there is highly efficient reductive detoxification of endogenously produced cytotoxic NO to N_2O, which then escapes from the cell. From this, it can be estimated that up to 20 % of nitrate catabolized is converted into the NO, which represents a substantial scale of intracellular production and thus requires very efficient detoxification of the cytotoxin.

N_2O production was maximal under nitrate-rich culture conditions where both *nar* and *nap* were expressed. However, mutagenesis confirmed that Nar was the major enzymatic route for the nitrate catabolism associated with N_2O production. (Figure 4 and Table 2). In the 20 h following anoxia, nitrate was consumed and nitrite accumulated in a near-stoichiometric fashion and very little N_2O accumulated (Figure 1B). The likely biochemical processes in operation are first nitrate import via the nitrate/nitrite antiporter NarK [32], the gene for which was also up-regulated during nitrate catabolism under nitrate-sufficient conditions. This is then followed by reduction to nitrite by NarG, and export of nitrite by NarK in exchange for incoming nitrate (Figure 7A). This process generates protonmotive force for growth. It is notable that, under these nitrate-rich culture conditions, the consumption of the nitrite produced from nitrate respiration is minimal and there is extensive extracellular accumulation of nitrite rather than the further reduction to ammonium (Figure 1B) despite expression of both *nirB* and *nrfA* (Figure 7A). Thus, although *Salmonella* Typhimurium is considered to be a canonical DNRA organism, under these nitrate-rich conditions, nitrite, rather than ammonium, is the major extracellular end-product of nitrate respiration. It makes sense from a bioenergetic view point to maximize nitrate respiration and minimize nitrite respiration under electron acceptor-sufficient conditions. This is because the $\uparrow H^+/e^-$ stoichiometry for reduction of nitrite by NrfA (2 with NADH as electron donor and 0 with quinol as electron donor) is lower than for nitrate reduction by Nar (3 with NADH and 1 with quinol) [33]. In this respect, it also makes bioenergetic sense to utilize the NarG nitrate reductase system, rather than the Nap system, since the $\uparrow H^+/e^-$ coupling ratios for periplasmic reduction of nitrate by Nap (2 with NADH and 0 with quinol) are also less than for Nar. This is highlighted by the lower biomass yield when the mutant in *narG*, that is dependent on Nap for growth, was cultured under the nitrate-sufficient conditions (Table 2).

At the end of the transition phase of the anoxic nitrate-sufficient continuous culture, the nitrite reaches an extracellular level that is $\sim$3-fold higher than that of nitrate (Figure 1). It is under these conditions that the rate of N_2O production is maximal. The rate of N_2O production matched the rate of nitrite reduction and the levels produced accounted for the balance of nitrate-nitrogen that did not accumulate as nitrite-nitrogen. The very low rate of N_2O production in the *narG* mutant suggested that it was linked to nitrite reduction by NarG. Competition between two substrates (nitrite and nitrate) for a single active site can be described by:

$$v_{nitrite}/v_{nitrate} = (k_{cat}/K_m)_{nitrite}/(k_{cat}/K_m)_{nitrate} \times ([nitrite]/[nitrate]) \text{ (adapted from [34])}$$

From the steady-state chemostat fluxes (Table 2), $v_{nitrite}/v_{nitrate} = qcNO_2^-/qcNO_3^- = 0.226$. From the kinetic parameters derived from the purified NarGH, $(k_{cat}/K_m)_{nitrite}/(k_{cat}/K_m)_{nitrate} = 0.007$. Thus [nitrite]/[nitrate] = 32 and this equates to the steady-state intracellular ratio of the two substrates. Such a ratio is perfectly conceivable if some of the nitrite exported by NarK in exchange for incoming nitrate re-enters the cell, possibly via by the bidirectional nitrite channel NirC [31], the gene for which was expressed under these growth conditions (Figures 2

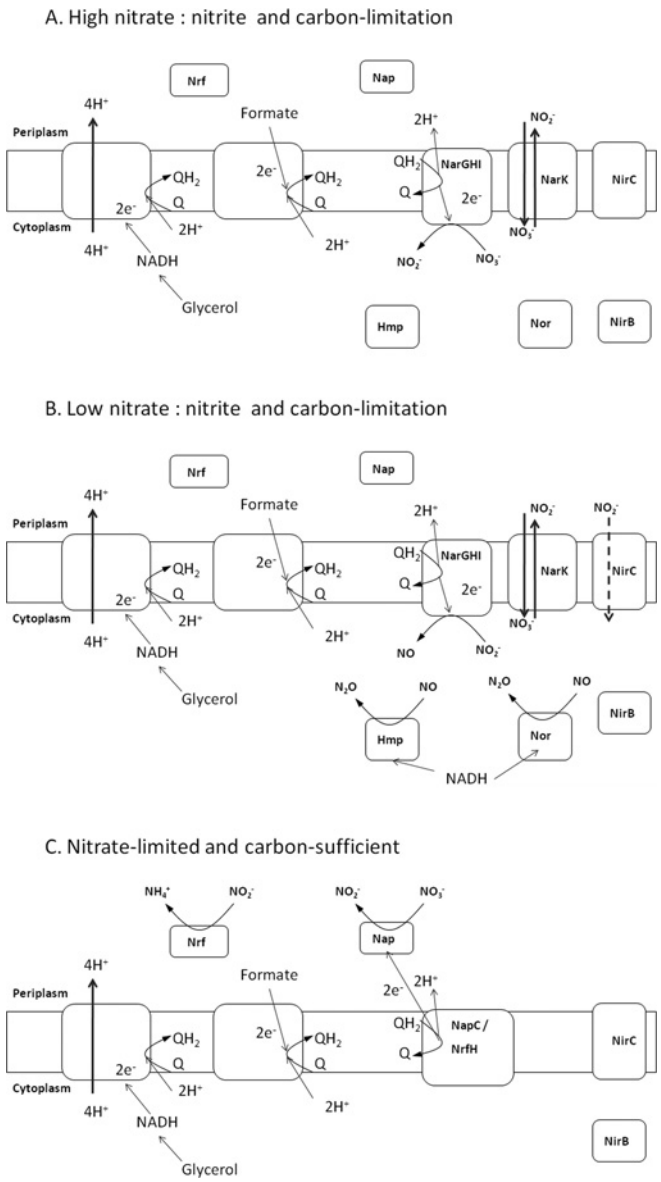

Figure 7 Schemes for nitrate and nitrite metabolism in *Salmonella* Typhimurium SL1344 under the different growth conditions explored in the present study

(**A**) In electron-acceptor-rich high-nitrate/nitrite conditions. Nar reduces nitrate to nitrite which is exported via NarK to the periplasm where it accumulates almost stoichiometrically with the nitrate consumed. (**B**) In electron-acceptor-rich low-nitrate/nitrate ratios, nitrite is imported by NirC and consumed via Nar producing NO, which is detoxified by Hmp and NorV to produce N_2O. (**C**) Under nitrate-limiting growth conditions, Nar is not synthesized, and the Nap and Nrf systems are actively consuming nitrate and the nitrite produced from nitrate reduction (with electrons flowing via the NapC and NrfH quinol dehydrogenases). No intracellular NO is generated, *hmp* and *norV* expression is low and N_2O is not produced.

and 7B). Intracellular nitrite may then out-compete intracellular nitrate for the active site of a NarG. This argument is supported by the experiments with the NarG mutant under nitrate-sufficient conditions that showed a near-stoichiometric consumption of nitrate and production of nitrite, and a very low level of N_2O production in the steady state (Figure 4). It is also consistent with the data of the early 1980s that led to the conclusion that NarG might reduce nitrite to N_2O and with more recent genetic and microbiological data that suggested that NarG in fact reduces nitrite to NO [3,11,12,18–20].

Under nitrate-limited growth conditions, the production of N_2O was very low in the steady-state phase, where both nitrate and nitrite were only present at low-micromolar levels. These conditions promoted expression of *napA* and *nrfA*, but not *narG* (Figure 2). This is consistent with the expression pattern observed in *E. coli* under low-nitrate/nitrite growth conditions [22,23,35–37]. Under these conditions, the absence of nitrite accumulation reflects that it is fully reduced through to ammonium by NrfA (Figure 7C, Reaction 2), so that there is maximum utilization of the limited respiratory electron-acceptor pool available to the culture. Thus results of the present study for *Salmonella* Typhimurium confirm observations from *E. coli* that when nitrate is sufficiently abundant, the bacteria exploit the energy-efficient, but low-affinity, NarG enzyme to reduce nitrate in the cytoplasm [22,23,35–37]. When nitrate is scarce, Nap provides a higher-affinity, but more poorly coupled, pathway that does not require nitrate transport for nitrate to serve as an effective electron sink [21] (Figure 7C).

Broadening the implications of the results more widely for the many species of Enterobacteriaceae that synthesize both NarG and NapA, the primary role of NarG is to generate protonmotive force when nitrate is abundant, such as occurs in nitrate-rich carbon-limited soils and sediments or wastewater-treatment plants [2]. Under these conditions, it has previously been thought that nitrite reductase NirB protects the cytoplasm from nitrite toxicity. However, the results of the present study show that nitrite can accumulate to millimolar levels in the extracellular medium and that, in fact, what nitrite is consumed is reduced to N_2O (Reactions 3 and 4) not ammonium (Reaction 2). However, in many habitats, Enterobacteriacaea will encounter much lower concentrations of nitrate, where the periplasmic pathway for nitrate and nitrite reduction is active [2]. We have shown that this combination will not lead to intracellular NO production, as judged by the lack of N_2O production and the absence of up-regulation of the *hmp* and *hcp* genes of NsrR regulon. The comparative experiments conducted with the *narG* and *napA* mutants demonstrate that nitrate reduction by Nap does not lead to extensive N_2O production even under nitrate-sufficient conditions.

Comparison of the *narG* and *napA* strains revealed a higher cell yield using Nar rather than Nap, reflecting the higher $\uparrow H^+/e^-$ for quinol oxidation by nitrate for Nar (1) compared with Nap (0) [33]. When Nap is operational, growth is dependent on energy-conserving formate dehydrogenase and NADH dehydrogenase reactions associated with glycerol metabolism for energy conservation, with *nap* serving a quinol pool recycling role [33]. However, although it makes bioenergetic sense to use NarG, under nitrate-rich conditions, there is a downside, which is the risk of cell damage associated with cytotoxic NO production. Co-ordinate induction of a NO-detoxification system minimizes this risk. There is, however, an energetic cost in using electrons in the non-energy-conserving cytoplasmic reduction of NO. The overall reduction of nitrite to N_2O via NarG and then HmpA or NorV using NADH consumes 2 mol of NADH (4 mol of e^-; Reactions 3 and 4) and yields an overall $\uparrow H^+/e^-$ of 1.5, which compares with an overall $\uparrow H^+/e^-$ of 2 when NADH is used via the respiratory electron-transport chain to reduce nitrite to ammonium. Thus it merits reflection on whether the diversion of $\sim$20 % of nitrate into NO and thence N_2O has any physiological importance. N_2O is a neuropharmacological agent that inhibits a range of cell receptors and transporters. The significance of N_2O production by pathogens has not been addressed, but it is likely to be a property of many enterobacterial pathogens and also pathogens such as *Neisseria* that have truncated denitrification pathways in which N_2O is the product of nitrite reduction due to the lack of N_2O reductase [38,39]. Further studies on the significance of N_2O production by pathogenic bacteria are therefore merited.

AUTHOR CONTRIBUTION

Daniela Hensen designed and executed the continuous culture experiments. Heather Felgate and Anke Arkenberg contributed to the continuous culture experiments. Corinne Appia-Ayme undertook the qRT-PCR experiments. Karen Prior and Carl Harrington constructed some of the mutants. Sarah Field undertook the enzyme kinetics. Julea Butt and Elizabeth Baggs contributed to the experimental concepts and discussion of the results. Gary Rowley and David Richardson designed the experiments and wrote the paper.

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