Nitric oxide (NO) is generated in biological systems primarily via the activity of NO synthases and nitrate and nitrite reductases. Here we show that Salmonella enterica serovar Typhimurium (S. typhimurium) grown anaerobically with nitrate is capable of generating polargraphically detectable NO after nitrite (NO$_2^-$) addition. NO accumulation is sensitive to the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Neither an fnr mutant nor an fnr hmp double mutant produces NO, indicating the involvement in NO evolution from NO$_2^-$ of protein(s) positively regulated by FNR. Contrary to previous findings in Escherichia coli, we demonstrate that neither the periplasmic nitrite reductase (NfrA) nor the cytoplasmic nitrite reductase (NirB) is involved in NO production in S. typhimurium. However, mutant cells lacking the membrane-bound nitrate reductase, NarGHI, and membranes derived from these cells are unable to produce NO, demonstrating that, in wild-type S. typhimurium, this enzyme is responsible for NO production. Membrane terminal oxidases cannot account for the NO levels measured. The nitrate reductase inhibitor, azide, abrogates NO evolution by Salmonella, and production of NO occurs only in the absence of the assays of nitrate; both features reveal a marked similarity between the NO-generating activities of this bacterium and plants. Unlike the situation in E. coli, an S. typhimurium hmp mutant produces NO both aerobically and anaerobically. Under aerobic conditions, when a functional flavohemoglobin is present, no NO is detectable. We propose a homeostatic mechanism in S. typhimurium, in which NO produced from NO$_2^-$ by nitrate reductase derepresses Hmp expression (via FNR and NsrR) and NorV expression (via NorR) and thus limits NO toxicity.

Salmonella enterica serovar Typhimurium (Salmonella typhimurium) survives and proliferates within macrophages, where it withstands anti-microbial responses such as the production of reactive oxygen species (1) and reactive nitrogen species, including nitric oxide (NO)\(^2\) (2). Enterobacteria possess several NO-detoxifying mechanisms, the most prominent being the flavohemoglobin Hmp (3–6) and the flavohemoglobin NorV (7). These enzymes detoxify incoming NO both aerobically (Hmp) and anoxically (NorV), converting the toxic gas to NO$_3^-$ or N$_2$O, respectively (8).

Conversely, certain bacteria produce NO. This is well documented in denitrifiers, where NO$_2^-$ is reduced to NO by the copper or cytochrome cd$_1$ nitrite reductase (9). Surprisingly, a number of enteric bacteria also produce NO when grown under NO$_3^-$-respiring conditions (10). Specifically, both S. typhimurium and Escherichia coli produce NO (10, 11), although the physiological significance of this is unclear. In E. coli, it has been reported that periplasmic nitrite reductase (NrfA; pentaheme periplasmic cytochrome c nitrite reductase) has a dual role in NO homeostasis; not only is it responsible for production of NO (11) but is also capable of detoxification of NO to NH$_4^+$ or N$_2$O anaerobically (12, 13). However, the major role of NrfA in E. coli is as a catalyst of the 6-electron reduction of NO$_2^-$ to NH$_4^+$ (14). Expression of NrfA is positively regulated at the transcriptional level by FNR under anaerobic conditions and is further enhanced by the presence of NO$_3^-$ and NO$_2^-$ via the NarL and NarP regulators. It has been suggested that there is an additional factor (15) exhibiting negative regulation over nrfA, which may be the NO-responsive regulator, NsrR (16, 17). The NO-consuming flavohemoglobin, Hmp, consumes endogenously produced NO, preventing inactivation of FNR (18), thereby preventing transcription of the FNR regulon that includes nrfA.

There is no evidence for nitric oxide synthase (NOS) activity in either E. coli or Salmonella, but several bacteria, including Nocardia (19), Staphylococcus aureus (20), Helicobacter pylori (21), Deinococcus radiodurans (22), Bacillus subtilis (23), and Streptomyces (24), do possess genes encoding NOS-like proteins. Evidence for NO production by purified B. subtilis NOS has been obtained using the mammalian neuronal NOS reductase domain as electron donor (23). The crystal structure of B. subtilis NOS complexed with L-arginine has confirmed the similarity between bacterial NOS and mammalian NOS (25). Interestingly, the nos gene of Streptomyces species has been identified on a pathogenicity island that confers the ability of the species to produce thaxtomin, a depectide phytotoxin required for plant pathogenicity. Therefore, Streptomyces NOS has been implicated in the nitration of thaxtomin (24).

The ability of bacteria to perform nitrosation reactions is well documented. In the stomach, bacteria catalyze formation of N-nitroso compounds from NO$_2^-$ at neutral pH values (26), and the resulting N-nitrosamines have been implicated in gastric cancer. Bacterial nitrosation has been characterized in vitro, using 2,3-diaminonaphthalene (DAN) as substrate and moni-
were incubated overnight in LB containing 100 mM NO₃⁻. Luria Broth (LB) containing potassium nitrate (KNO₃) at a final concentration of 100 mM, supplemented as appropriate with kanamycin (50 μg/ml), tetracycline (25 μg/ml), or chloramphenicol (Cm) (25 μg/ml). Anaerobic cultures for NO production were grown for ~24 h in 1-liter Duran bottles filled to the brim with media (LB containing 100 mM KNO₃ and 5% glycerol). Cells were then harvested by centrifugation at 5000 rpm, 4 °C. The supernatant was removed, and the cells were resuspended in 1 ml of 10 mM, final concentration) was added, and the tubes were incubated for a further 18 min. Cells were pelleted by brief centrifugation, and the cells were resuspended in ~300 μl of the supernatant. Aliquots (100 μl) of the transduction mixture were then plated onto NA supplemented with Cm to a final concentration of 25 μg/ml. Putative mutants were picked the following day and verified by PCR amplification of the nirA region. The mutation was transduced into a clean wild-type background using P22 (34), selecting for CmR. The CmR marker was also inserted into the nirB gene using the same methods.

**P22 Transduction**—Lysates were prepared as described previously (34). Overnight cultures of strains were grown in LB. Aliquots (100 μl) of the recipient were then mixed with 10 μl of donor lysate for 12 min at 37 °C. LB (0.9 ml) containing EGTA (10 mM, final concentration) was added, and the tubes were incubated for a further 18 min. Cells were pelleted by brief centrifugation, and the cells were resuspended in ~300 μl of the supernatant. Aliquots (100 μl) of the transduction mixture were then plated onto NA supplemented with Cm to a final concentration of 25 μg/ml. After an overnight incubation, any putative transductant colonies were re-streaked, and the mutation was confirmed by PCR.

**Western Blots**—Western blots were carried out exactly as described previously (6).

**Preparation of Periplasmic Fractions**—Periplasmic extracts were made exactly as described previously (11).

**Preparation of Membranes**—Anaerobic cultures were grown for ~24 h in 1-liter Duran bottles filled to the brim with media (LB containing 100 mM KNO₃ and 5% glycerol). Cells were then harvested by centrifugation at 5000 × g for 20 min at 4 °C. Cells were resuspended in 10 ml of membrane buffer (50 mM Tris-HCl containing 2 mM MgCl₂ and 1 mM EGTA, pH 7.5) and lysed by French pressing. Debris was pelleted by centrifugation at 12,000 × g for 15 min at 4 °C and the supernatant (containing membranes) transferred to an ultracentrifuge tube. Crude extracts were ultracentrifuged for 1 h at 225,000 × g, 4 °C. The membrane pellet was washed in membrane buffer and ultra-centrifugation was repeated. The final membrane pellet was resuspended in 1 ml of buffer.

**Cytochrome Assays**—Difference spectra (CO + reduced minus reduced) of washed membranes were recorded essentially as described (35) in a Johnson Foundation SDB3 dual-wavelength scanning spectrophotometer at room temperature. Samples were reduced with dithionite, bubbled with CO for 2.5 min, and used to record spectra in cells of 10-mm path length. After treatment with CO, samples were scanned successively until no further CO-induced changes were observed to allow for slow CO reactivity in such anoxically grown cells. Other scan conditions were as described before (35). For quantifying cytochrome bo', we used ε = 145 mM⁻¹ cm⁻¹ (416–430 nm) (36). Cytochrome c₅₅₂ concentrations in periplasmic fractions were determined spectrophotometrically as described previously (11).

| Bacterial strains used in this study | S. typhimurium | Source/Ref. |
|-------------------------------------|---------------|------------|
| Strains                             |               |            |
| 14028s lmu::kan⁸                     | Wild-type     | 3          |
| 14028s lmu::kan³                     | lmu::kan³     | 3          |
| 14028s for::tet⁸                     | for::tet⁸     | S. Grogan, University of Sheffield |
| 14028s for::tet³, lmu::kan³          | for::tet³, lmu::kan³ | This study |
| 14028s nirA::Cm⁶                     | nirA::Cm⁶     | This study |
| 14028s nirB::Cm⁶                     | nirB::Cm⁶     | This study |
| SL1344                               | xyl hisG rpsL | A. Stevenson, University of Glasgow |
| SL1344 narGHIJ::kan⁶                 | narGHIJ::mudj | 75         |
| Strains                             |               |            |
| SL1344 for::tet⁶                     | for::tet⁶     | S. Grogan, University of Sheffield |
| SL1344 narGHIJ::kan³                 | narGHIJ::mudj | 75         |

**TABLE 1**

Bacterial strains used in this study

- Strains
- S. typhimurium
- Source/Ref.

- Strains
- 14028s lmu::kan⁸
- 14028s lmu::kan³
- 14028s for::tet³
- 14028s for::tet³, lmu::kan³
- 14028s nirA::Cm⁶
- 14028s nirB::Cm⁶
- SL1344
- SL1344 narGHIJ::kan³

| Bacteriophage | Plasmids |
|---------------|----------|
| P22           | pTP223   |

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Culture Conditions**—Table 1 lists strains used in this study. Anaerobic cultures were grown at 37 °C in Luria Broth (LB) containing potassium nitrate (KNO₃) at a final concentration of 100 mM, supplemented as appropriate with kanamycin (50 μg/ml), tetracycline (25 μg/ml), or chloramphenicol (Cm) (25 μg/ml). Anaerobic cultures for NO evolution experiments and Western blots were grown in 16-ml screw-cap glass tubes filled to the brim with media. Cultures were incubated overnight in LB containing 100 mM NO₃⁻ (two tubes for each strain). Cells were harvested by centrifugation (10 min, 5500 rpm, 4 °C) and washed with phosphate-buffered saline (PBS), pH 7.4, and then suspended in PBS to a final volume of 1.0 ml. Cells for Western blots were resuspended in 1 ml of 50 mM Tris, pH 7.5. Anaerobic cultures for cytochrome c₅₅₂ assays were grown in 500-ml Duran bottles, filled to the brim (total volume 620 ml), for 24 h to stationary phase of growth.

**Mutagenesis**—The λ Red system was used to promote replacement (first described in E. coli (33)) of a large portion of the nirA gene with a Cm resistance (CmR) gene. The CmR gene from pACYC184 was PCR-amplified with primers having 40 bp of both 5’- and 3’-flanking complementarity to the S. typhimurium nirA gene. The linear DNA fragment was electroporated into wild-type S. typhimurium carrying pTP223 and transformants selected on nutrient agar containing Cm (final concentration 25 μg/ml). Putative mutants were picked the following day and verified by PCR amplification of the nirB region. The mutation was transduced into a clean wild-type background using P22 (34), selecting for CmR. The CmR marker was also inserted into the nirB gene using the same methods.

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NO Production by Salmonella, Involvement of Nar

Protein Assays—The protein contents of intact cells were measured using the protocol of Ref. 37. The protein contents of membranes, periplasmic fractions, and cleared supernatant fractions from sonicated cells were measured using the Bio-Rad protein assay kit and bovine serum albumin as the standard.

NO Evolution and O₂ Measurements—Concentrations of dissolved oxygen and NO were measured in a Clark-type polarographic oxygen electrode system (Rank Bros., Bottisham, Cambridge, UK) modified to accommodate a World Precision Instruments ISO NOP sensor (2-mm diameter) (38). Cell suspension was diluted in the chamber (final volume 2 ml) with PBS buffer. Sodium formate (final concentration 25 mM) was used to induce rapid depletion of oxygen in preparation for anaerobic experiments, and a final concentration of 5 mM was used to allow slower reduction of oxygen in aerobic NO evolution experiments. Membrane suspensions were diluted in the chamber in membrane buffer, and 6 mM NADH (final concentration) was added to induce rapid oxygen uptake. For anaerobic NO evolution experiments, a close-fitting lid, with a fine hole for injections using a Hamilton syringe, was inserted. When respiration had reduced oxygen to the required level, the suspension was supplemented with an anoxic solution of NaN₃ to a final concentration of 25 mM, and NO evolution was then measured. NaN₃ solutions were made anoxic by bubbling the solution with argon for 20–30 min. For aerobic NO evolution experiments, an open electrode chamber was used, in which the stirred sample was open to the atmosphere, allowing continuous oxygen diffusion from the vortex surface into the sample, and prolonged measurements to be made without oxygen depletion. The O₂ transfer constant K was determined from the observed half-time of the first-order equilibration of liquid (made anoxic with a few grains of sodium dithionite) with atmospheric oxygen (39). A value of $K = 0.36 \text{ min}^{-1}$ was assumed under these conditions of temperature and stirring rate. The O₂ electrode was calibrated using air-equilibrated PBS and the addition of sodium dithionite (a few grains) to achieve anoxia. The NO electrode was calibrated by addition of known concentrations of NaN₃ to an acidified potassium iodide solution as described by the manufacturer. Additions of anoxic, NO-saturated solutions, NaN₃ and 3.75 mM c-PTIO (Calbiochem), were made using Hamilton syringes. NO was generated exactly as in Ref. 40. A stock solution of 0.5 M NaN₃ was freshly made each day in a bottle fitted with a rubber septum (“Suba-seal,” VWR International); argon gas was bubbled through the solution for 20–30 min, making it anoxic.

RESULTS

NO Production by S. typhimurium and the Role of Hmp in NO Homeostasis—S. typhimurium strains were grown anaerobically overnight in the presence of 100 mM NO₃⁻. S. typhimurium wild-type and hmp mutant strains grew similarly in these conditions (data not shown), whereas in E. coli growth of the hmp mutant is poor compared with the wild-type strain (11). Cells were harvested, resuspended in PBS, and added to oxygenated PBS in a closed oxygen electrode chamber with formate as electron donor. When oxygen levels fell to zero, NaN₃ was added (final concentration 25 mM), and NO evolution was followed. Fig. 1A shows a typical NO production trace for the 14028s wild-type strain; NO production was observed for 10–30 min before the levels of NO reached a plateau, typically at around 30 μM, but small daily variations were seen (Fig. 1B). Cultures grown anaerobically in the presence of 50 mM fumarate in place of NO₃⁻ were unable to produce NO from NO₃⁻ (Fig. 1B), indicating that the factor responsible for NO evolution is not expressed under these growth conditions. NO₃⁻ (12.5 mM) also caused NO evolution to similar levels (Fig. 1C). Lowering the concentration further to 6.25 mM NO₃⁻ led to a disproportionate decline in NO evolution (Fig. 1C). The small rise in oxygen levels recorded (Fig. 1A) during the experiment is attributed to

![FIGURE 1. NO production in S. typhimurium and the relationship with flavohemoglobin. A, anaerobic NO production by wild-type strain 14028s. Fine line represents oxygen and the bold line NO. Total protein content for cell suspension was 6.6 mg/ml. B, plateaus levels of NO produced (means ± S.D.) under anaerobic conditions following growth in the presence of 100 mM NO₃⁻ or 50 mM fumarate (fum) by 14028s wild-type (WT), hmp, and fnr mutant strains. C, NO evolution from wild type following addition of varying concentrations of NO₂⁻. Upward facing arrows below the x-axis represent NO₂⁻ additions. Downward facing arrows represent additions of c-PTIO (3.75 mM). Fine black trace, dashed trace, and thick black trace represent NO evolved following addition of 25, 12.5, and 6.25 mM NO₂⁻, respectively. Data shown here in A and C represent averages of at least three biological replicates. Data presented in B are means ± S.D. of at least three biological replicates.](image-url)
oxygen diffusion into the chamber through the small injection holes. To verify that the response seen by the ISO-NOP electrode was because of production of NO, the NO scavenging compound, c-PTIO, was added to the chamber; a rapid fall in NO was sensed by the electrode (Fig. 1, A and C). In contrast with E. coli, the S. typhimurium hmp mutant was able to produce NO under anaerobic conditions with very similar NO production profiles to the wild-type strain (Fig. 1B).

Because of the key roles of FNR in activating the pathways of anaerobic respiration, we tested an fnr mutant for its ability to generate NO from NO₂⁻ under identical conditions to those shown in Fig. 1A; NO production did not occur (Fig. 1B). As FNR is a negative regulator of hmp transcription (18, 40, 41), one reason for lack of NO production may be high levels of NO detoxification by Hmp, albeit under the anoxic conditions used for the assays (38, 42, 43). To determine the effects of FNR on Hmp levels, Western blot analysis was carried out on wild-type, hmp, and fnr mutant strains following overnight growth of anaerobic cultures in LB containing 100 mM NO₃⁻. Higher levels of Hmp expression in the fnr mutant compared with the wild-type strain were confirmed (data not shown).

When NO evolution assays were carried out in the presence of oxygen, i.e. under conditions where Hmp is most active (38), the wild-type strain did not show NO production (data not shown). These results suggest that wild-type levels of Hmp are sufficient to prevent aerobic NO accumulation. Note that under the conditions used for growth, i.e. anoxically in the presence of NO₃⁻, hmp transcription is markedly up-regulated (40). To investigate the role of Hmp, we next assayed NO production in an hmp mutant. In these experiments, an “open” electrode system was used, and addition of 25 mM NO₂⁻ was made at ~25% of saturated oxygen levels. Under these conditions, a rapid phase of NO production was observed on NO₂⁻ addition (Fig. 2). Indeed, the concentration of NO evolution by the hmp strain aerobically was over 10-fold greater than anaerobically (Fig. 1B). We attribute this to the combined effect of (i) the lack of NorV activity aerobically (44) and (ii) the deletion of Hmp. Because the electrode system was open to the atmosphere, the abrupt inhibition of respiration by the added NO₂⁻ or the NO formed caused an increase in the steady-state O₂ level, as evidenced by the sharp rise in the O₂ trace (Fig. 2). Approximately 2–3 min after the detection of NO, the NO electrode signal declined, presumably because of nonenzymic reaction with O₂ whose concentration rises during this period (Fig. 2). Collectively, these results demonstrate that aerobically, but not anaerobically, Hmp activity is sufficient to consume the NO generated by NO₂⁻ reduction; however, these experiments do not identify the reductase responsible.

**Identification of FNR-activated NO-generating Systems**—To test the hypothesis that enhanced NO detoxification by Hmp in the fnr mutant strain prevents detection of NO production from the fnr mutant, an fnr hmp double mutant was created by P22 transduction of the hmp mutation into the fnr mutant strain. Transfer of the mutation was confirmed by Western blot analysis (data not shown). Results showed that, even in the absence of hmp, NO production could not be detected from the fnr mutant (data not shown). This clearly indicates that it is not enhanced levels of Hmp that cause the fnr mutant strain to lack the observed NO-producing ability but rather the failure in the mutant to express the NO-producing protein. Previous work with E. coli (11) suggested this to be nitrite reductase, specifi-
Nitrate Reductase, NarGHI, Is Involved in Conversion of NO$_3^-$ to NO—Plant nitrate reductases are known to convert NO$_3^-$ to NO (45). We therefore asked whether S. typhimurium nitrate reductase may be able to carry out similar chemistry. An S. typhimurium SL1344 strain with a mutation in the narGHJI operon was unable to produce NO (Fig. 3A). This strongly indicates that the Salmonella membrane-bound nitrate reductase, NarGHI, which is positively regulated by FNR in the absence of oxygen, and by phosphorylated NarL in the presence of NO$_3^-$, is responsible for NO production. To test whether the nitrite reductase, NrfA, is expressed at wild-type levels in the narGHJI mutant, the periplasmic levels of cytochrome $c_{552}$ in the Salmonella strains were assayed spectrophotometrically (Fig. 3B). Reduced minus oxidized difference spectra revealed peaks in the Soret (422 nm) and $\alpha$-regions (552 nm) indicative of cytochrome $c_{552}$. Signal intensities adjusted for protein content were similar in periplasmic fractions from both wild-type and narGHJI mutant strains grown anaerobically in the presence of 100 mM NO$_3^-$. Thus, NrfA is expressed similarly in both strains, despite the inability of the narGHJI mutant to reduce NO$_3^-$ to NO$_2^-$. To further implicate NarGHI in the production of NO from NO$_2^-$, an inhibitor of nitrate reductase, sodium azide (46), was used (Fig. 3C). Immediately following azide addition, NO sensed by the electrode dramatically fell; when azide was added to cells immediately prior to NO$_3^-$, NO production was not observed (data not shown).

Previous work on nitrate reductases from several plant species has shown that NO$_3^-$ is the preferred substrate and that nitrate reductase cannot simultaneously produce NO and reduce NO$_2^-$ (47). We investigated whether this is the case in S. typhimurium. An addition of NO$_3^-$ was made to the electrode chamber while wild-type cells were producing NO. Fig. 4A shows that, immediately following NO$_3^-$ addition, NO rapidly fell. This reveals that the balance between NO production and NO consumption moves toward NO consumption by NorV, NrfA, and/or Hmp. Next, we made additions of NO$_3^-$ to cells in the chamber, immediately prior to NO$_2^-$ addition. When 0.5 mM NO$_3^-$ was added prior to 25 mM NO$_2^-$, a lag of $\sim$2 min was seen between addition of NO$_3^-$ and NO production (Fig. 4B). When the amount of NO$_3^-$ added was halved to 0.25 mM, the lag phase was also approximately halved to 1 min between NO$_3^-$ addition and NO production (Fig. 4C). To confirm that added NO$_3^-$ does not per se interfere with NO detection, we generated NO in the chamber with 3-[(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC-7), an NO-generating agent that releases NO with a half-life of 10 min at pH 7.4, 22 °C] and showed that subsequent addition of NO$_3^-$ did not quench the electrode signal (inset in Fig. 4A). These results indicate that, in the presence of NO$_3^-$, the nitrate reductase,
NarGHI, reduces NO$_3^-$ to NO$_2^-$, but when NO$_2^-$ is exhausted, the enzyme switches to NO production from NO$_3^-$.

The above studies with nir, nrf, and nar mutants clearly point to a role for nitrate, but not nitrite, reductases in NO production. As confirmation that soluble nitrite reductases were not required, membranes were prepared from wild-type SL1344 and narGHIJ mutant strains grown anaerobically for ~24 h in LB containing 100 mM NO$_3^-$ and 5% glycerol. Membrane fractions were used in the oxygen electrode chamber as cell suspensions were previously; 25 mM NO$_2^-$ was added to the membranes when the O$_2$ in the chamber became zero. Respiration by the membranes was stimulated by the addition of azide, KNO$_3$, or c-PTIO between 20 and 30 min. Bold line represents NO production by narGHJU mutant membranes. Total protein content was 8.1 and 7.1 mg/ml, respectively, for wild-type and narGHJU membrane suspensions. B, CO difference spectra of membranes used in A. For details see text.

**DISCUSSION**

In this paper, we demonstrate that anaerobically cultured S. typhimurium generates NO from NO$_3^-$ under anoxic conditions. These data share features with an earlier study of E. coli (11), summarized in Table 2. In both organisms, mutation of the oxygen-responsive transcription factor FNR eliminates NO generation, because of loss of up-regulation of the structural gene(s) encoding the reductase responsible for NO generation. However, in marked contrast to the parallel situation in E. coli, elimination by mutation of either nitrite reductase (NrfA or NirB) has no effect on NO release in Salmonella. A further difference between the two studies is the effect of mutation of hmp encoding the NO-detoxifying flavohemoglobin. Whereas in E. coli, an hmp mutant is defective in NO generation, an hmp mutation has no effect in Salmonella. The effect of an hmp mutation correlates with the nitrite reductase data; loss of Hmp in E. coli is correlated with decreased levels of the NO-generating nitrite reductase (cytochrome c$_{552}$). We have previously suggested (11) that NO accumulation in the hmp mutant inactivates FNR (18) with loss of up-regulation of the nitrite reductase responsible for NO generation. The present finding that mutation of hmp is without effect on anaerobic NO generation is consistent with the view that Hmp has primarily an O$_2$-dependent NO-detoxifying role (51). Under aerobic conditions, however (Fig. 2), mutation of Hmp allows accumulation of NO.

However, the most important finding of this study is that nitrate reductase (NarGHI) is responsible for NO generation from NO$_3^-$ in Salmonella. As in plants, NO generation was inhibited by NO$_3^-$ in competition with NO$_2^-$ as substrate and by azide. Most significantly, mutation of narGHIJ eliminates all detectable NO release in both cells and derived membranes. More direct biochemical evidence for the involvement of nitrate reductase might be possible with studies of the purified enzyme, but most mechanistic studies of the enzyme have been performed with membranes, as here (52). To avoid the problems of using short chain water-soluble quinols or quinol analogues as artificial donors and to obviate changes in protein activity on solubil and purification, we exploited washed membranes and the availability of an narGHJ mutant. Although the possibility remains that other oxidoreductases might contribute to NO evolution, neither E. coli nor Salmonella possesses the cytochrome cd$_1$ nitrite reductase, which produces NO and has been extensively studied in Pseudomonas.
aeruginosa (53). Other candidates are the terminal oxidases cytochromes bo’ and bd, both of which are detected in membranes from wild-type and narGHIJ mutant strains (Fig. 5B). However, the levels of both oxidases are unchanged in the narGHIJ mutant despite the inability to detect NO formation in this strain (Fig. 5A). Cytochrome bd has no reported NO2-reducing activity to our knowledge, nor does it reduce NO (54). Cytochrome bo’ is a member of the heme-copper superfamily of terminal oxidases and thus shares mechanistic properties with mitochondrial cytochrome c oxidase (cytochrome aa3).

Recently, it has been demonstrated that both yeast and mammalian oxidase produce NO from NO3 under anoxic conditions (55) similar to those used in this study. However, the accumulation levels of NO in assays employing mitochondria and Salmonella are very different. Using the data of Ref. 55 and assuming that accumulation of NO in yeast occurs over 10 min at the initial rate reported, we calculate that, in yeast mitochondria, NO accumulated is about 0.2 nmol of NO/mg of protein, compared with values at least 100-fold higher in bacteria (Table 2). Castello et al. (55) also provide data for NO generation by the purified yeast cytochrome c oxidase, which we calculate to be \(-3\) nmol of NO per mg of oxidase protein (if NO accumulates at the initial rate for 10 min). The cytochrome bo’ content of wild-type membranes is 0.17 nmol/mg membrane protein; assuming a molecular mass for the oxidase of 145 kDa (56), and that all NO measured in Fig. 5A arises from cytochrome bo’, we can calculate that the capacity of cytochrome bo’ for NO generation would be in the order of 1,900 nmol of NO per mg of oxidase protein. This exceeds the measured value for the mitochondrial oxidase by 600-fold, and we conclude that cytochrome bo’ is unlikely to make a significant contribution to this study.

This study reveals similarity between plant nitrate reductases and the S. typhimurium NarGHI nitrate reductase. The enzymes differ in their location, structural components, and role within the cell. The plant reductase is assimilatory and water-soluble, whereas the enterobacterial enzyme is involved in dissimilatory NO3 reduction during anaerobic respiration (57). Both eukaryotic and all bacterial nitrate reductases contain a molybdenum cofactor. All bacterial nitrate reductases contain a [Fe-S] cluster, but there are no known eukaryotic nitrate reductases that contain [Fe-S] centers (58). Membrane-bound nitrate reductase (NarGHI) has been characterized in many NO3 respiring and denitrifying bacteria. The Nar enzyme is composed of three subunits: NarG (catalytic molybdenum-containing subunit), NarH ([Fe-S]-containing electron transfer subunit), and NarI (heme-containing membrane anchor subunit) (59 and reviewed in Ref. 60). Earlier work (10, 27–30) indirectly implicated Nar in the production of NO by studying the nitrosating ability of E. coli. This study confirms the importance of nitrate reductase not only in its potential for nitrosation but directly in NO evolution.

Plant nitrate reductase is now well established as producing NO (45, 47, 61–63). Purified cytosolic nitrate reductase produces NO with NADH as the electron donor (45) and, like the work presented here, the reaction is azide-sensitive (63). NO3 is a competitive inhibitor of NO production in leaves, and NO production was dependent on a number of factors, including cytosolic NO3 and NO2 concentrations, light, oxygen availability, and serine phosphorylation of nitrate reductase (47). The role of NO production by plants has been widely discussed, and it is possible that low level NO production by nitrate reductase may have a role in cellular signaling, in particular in establishing symbiosis and in defense against pathogens (62). However, in planta, mutant studies have shown that...
NO\textsubscript{2} may be the major source of NO by *Arabidopsis thaliana*, when the plant is challenged with infection (61).

The physiological roles for the production of NO by *S. typhimurium* are still unclear. NO is generated within the acidic environment of the stomach in the presence of salivary NO\textsubscript{2}, and the concentration of NO in the stomach headspace can range from 20 to 400 ppm (26, 64). NO is also generated by probiotic bacteria in the gut (65), where it may have a beneficial effect, for example in stimulation of mucosal blood flow and peristalsis (66). However, an excess of NO during infection may modulate host signaling pathways that cause deleterious effects in the host. For example, NO can cause dilation of tight junctions between gut epithelial cells (67, 68), which may allow easier access of *S. typhimurium* into the lower layers of the gut. NO may also be produced to aid killing of commensal organisms in the gut.

Fig. 6 draws on evidence from this study and others and summarizes the homeostatic balance of NO production and consumption that occurs within *S. typhimurium*. In aerobic conditions, FNR forms active dimers and positively regulates transcription of many genes, including the *nrf*, *nir*, and *nar* operons (69). In the presence of NO\textsubscript{3}/NO\textsubscript{2}, NarL and/or NarP are phosphorylated to further enhance transcription of the *nrf* (70), *nir* (71), and *nar* (31) operons. NarGHI reduces NO\textsubscript{3} to NO\textsubscript{2} (60). NO\textsubscript{2} can be further reduced by NirB in the cytoplasm or NrfA in the periplasm. In the absence of NO\textsubscript{3}, NarGHI can reduce NO\textsubscript{2} to NO (this study). The presence of NO enhances the transcription of *hmp* through the inactivation of FNR (18, 40, 41) and/or NsrR (5, 6, 72). NorV is also expressed via activation of the positively acting transcription factor, NorR (73). During aerobic conditions Hmp is able to convert NO to NO\textsubscript{3} and anaerobically can reduce NO to N\textsubscript{2}O, albeit with greatly reduced activity (38, 43, 74). NorV is active anaerobically only and reduces NO to N\textsubscript{2}O. NrfA can also act anaerobically to detoxify NO to NH\textsubscript{3}O or N\textsubscript{2}O (12, 13). These mechanisms constitute a cooperative network that is likely to exist anaerobically between Nrf, Nar, and Nor to ensure complete reduction of NO\textsubscript{3} to a “safe” product.
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