Nitric Oxide Formation by *Escherichia coli*

DEPENDENCE ON NITRITE REDUCTASE, THE NO-SENSING REGULATOR Fnr, AND FLAVOHEMOGLOBIN Hmp

Hazel Corker and Robert K. Poole‡

From the Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, United Kingdom

Nitric oxide (NO) is a key signaling and defense molecule in biological systems. The bactericidal effects of NO produced, for example, by macrophages are resisted by various bacterial NO-detoxifying enzymes, the best understood being the flavohemoglobin exemplified by *Escherichia coli* Hmp. However, many bacteria, including *E. coli*, are reported to produce NO by processes that are independent of denitrification in which NO is an obligatory intermediate. We demonstrate using an NO-specific electrode that *E. coli* cells, grown anaerobically with nitrate as terminal electron acceptor, generate significant NO on adding nitrite. The periplasmic cytochrome c nitrite reductase (Nrf) is shown, by comparing Nrf⁺ and Nrf⁻ mutants, to be largely responsible for NO generation. Surprisingly, an *hmp* mutant did not accumulate more NO but, rather, failed to produce detectable NO. Anaerobic growth of the *hmp* mutant was not stimulated by nitrate, and the mutant failed to produce periplasmic cytochrome(s) c, leading to the hypothesis that accumulating NO in the absence of Hmp inactivates the global anaerobic regulator Fnr by reaction with the [4Fe-4S]²⁺ cluster (Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., Green, J., and Poole, R. K. (2002) *EMBO J.* 21, 3235–3244). Fnr thus failed to up-regulate nitrite reductase. The model is supported by the inability of an *fnr* mutant to generate NO and by the restoration of NO accumulation to *hmp* mutants upon introducing a plasmid encoding Fnr (D154A) known to confer activity in the presence of oxygen. A cytochrome bd-deficient mutant retained NO-generating activity. The present study reveals a critical balance between NO-generating and -detoxifying activities during anaerobic growth.

Nitric oxide (nitrogen monoxide, NO)³ is a molecule of major importance in biological systems where it plays signaling, vasodilatory, and cytotoxic roles. Recent attention has focused on NO synthesis from the sequential oxidation of l-arginine by NO synthases in eukaryotic cells (1), their mitochondria (2), and certain bacteria (3). NO is also an obligate intermediate in denitrification, the process by which certain bacteria sequentially reduce nitrate ion to dinitrogen (4, 5). However, several representatives of the “non-denitrifying” Enterobacteriaceae, including *Escherichia coli*, grown anaerobically with nitrate, were shown to produce up to one-twentieth of the NO produced by denitrifiers. NO production from nitrite, measured by the nitrosation of 2,3-diaminonaphthalene (DAN) (6) was proposed to involve enzymatic reduction of nitrite to NO followed by oxygen-dependent DAN nitrosation. NO production has also been shown in *Serratia marcescens* (7), *Bacillus cereus* (8), three species of methanotrophic bacteria (9), and the green micro alga *Scenedesmus obliquus* (10). Ji and Hollocher (11) concluded that nitrite-dependent NO production by *E. coli* was due to the activity of the membrane-associated (dissimilatory) nitrate reductase. Nitrate reductase exhibited at all stages of its purification a nitrite reductase activity, which was strongly inhibited by nitrate and azide.

More recent evidence for NO production by *E. coli* has come from expression of the *Paracoccus denitrificans* transcription factor NNR in *E. coli*. This protein is activated by NO, and transcription of a target *melR-lacZ* promoter in *E. coli* was attributed to formation of NO (or related species) from nitrate by molybdenum-dependent nitrite reductase (12). NO production from nitrite, however, was not dependent on molybdenum cofactor biosynthesis.

Since the initial reports of NO production by *E. coli* (6, 13), advances have been made that prompt a reinvestigation. First, sensitive NO electrodes with markedly improved selectivity have been developed (14). Second, several proteins have functions firmly linked to the stresses imposed by NO and nitrosation (15–17), yet the potential physiological stresses imposed by endogenously generated NO have not been assessed. The best understood is the flavohemoglobin, Hmp, of *E. coli*, encoded by a gene that is inducible under both aerobic and anaerobic conditions by nitrate, but especially nitrite and NO (18). NO consumption by Hmp aerobically generates nitrate (19–21), but, anaerobically, Hmp detoxifies NO by converting it to NO⁻ with N₂O appearing as a product (20, 22). The global aerobic-anaerobic regulators Fnr and MetR are involved in *E. coli* *hmp* regulation (15, 23). Third, certain bacteria (e.g., *Nocardia* (24)) have been shown to possess nitric-oxide synthases similar to those reported in certain plants (25) and mammalian systems, suggesting a role for NO in bacterial physiology. These findings suggest an interplay between NO-producing and NO-consuming activities of physiological relevance in non-denitrifying bacteria.

In this report, we provide direct and sensitive measurements of NO production by *E. coli* cells from nitrite and explore the
roles of Hmp and of cytochrome b(5) (previously shown to react with and reduce nitrate (26, 27)) in this process. We demonstrate the involvement of the nrfA-encoded cytochrome c nitrite reductase in generating NO and the lack of NO generation in mutants lacking the flavohemoglobin Hmp, the narG-encoded nitrite reductase, or the global regulator Fnr.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli K-12 strains used in this study are described in Table I.

Media and Culture Conditions—Cells were grown at 37 °C in Luria Broth (LB), pH 7.0 (32), supplemented as appropriate with kanamycin (100 μg/ml) or ampicillin (200 μg/ml). KNO3 was added as a filter-sterilized solution to LB at a final concentration of 40 or 100 mM as indicated. Aerobic cultures were grown with shaking (200 rpm) in side-arm flasks containing 170 ml of their own volume of medium. Anaerobic cultures (for NO evolution experiments) were grown in screw-cap glass tubes filled to the brim and containing a glass bead (~1-mm diameter) to aid resuspension of cells that had sedimented during static culture. 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. Cells were harvested by centrifugation and washed twice at 4 °C with phosphate-buffered saline (PBS), pH 7.3, then suspended in PBS to a final volume of 1.0 ml. Strains JCB352 and JCB387 were washed and resuspended in LB. A Kleiss-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, NY) equipped with a no. 66 (red) filter was used to monitor turbidity of cultures grown in screw-cap glass tubes or side-arm flasks. Culture density at 420 nm (A420) was measured using a Jenway 6100 spectrophotometer in cells of 1-cm pathlength.

Preparation of Periplasmic and Cytoplasmic Extracts—Periplasmic and cytoplasmic extracts were made using a modified version of earlier protocols (33, 34). Aerobic cultures (200 ml in a 1-liter conical flask, 200 rpm) were grown to an A420 of 0.5–0.6 (~4 h after inoculation) and then conditioned for osmotic shock by the addition of NaCl and Tris-HCl buffer (pH 7.3) to a final concentration of 30 mM each. Cells from 400 ml of culture were resuspended in 6 ml of a buffer containing (final concentrations) 33 mM Tris-HCl (pH 7.3), 20% (w/v) sucrose, and 1 mM sodium EDTA. After incubation for 20 min at room temperature, cells were collected by centrifugation at room temperature and then rapidly resuspended in 6 ml of ice-cold water. After mixing for 45 s on ice, Mg2+ was added to a final concentration of 1 mM, and the suspension was mixed on ice for 45 s, then left on ice for a further 10 min. This suspension was centrifuged at 10,000 × g for 5 min, and the resulting supernatant fraction (periplasm) was stored on ice prior to use. The remaining pellet was resuspended in a buffer that contained (final

![Fig. 1. NO evolution by E. coli is nitrite-dependent.](image-url) Measurements of NO production of washed cell suspensions (~0.82 mg of cell protein/ml) of E. coli wild-type strain AN2342, grown anaerobically in the presence of 100 mM KNO3, were made in an oxygen electrode apparatus as described under “Experimental Procedures.” In B, a solution of NaNO2 was added at the first arrow to give final concentrations of 25 mM NaNO2 (a), 12.5 mM NaNO2 (b), 5 mM NaNO2 (c), and 2.5 mM NaNO2 (d). The vertical bar corresponds to 35 μM NO in a and d, and 33 μM NO in b and c. The NO-reactive compound carboxy-PTIO, where added, is indicated by the letter C, and final concentrations (μM) are given in brackets. The experiment was repeated at least twice with similar results. The insets show O2 consumption on respiration of added formate (A, left) and NO2 dependence of steady-state NO concentrations achieved (C, right).
concentrations) 20% sucrose, 1 mM sodium EDTA, and 200 mM Tris-HCl (pH 7.5). Sonication (5 15-s bursts with 30-s intervals) was followed by high speed centrifugation (1.57 × 10^11 g for 70 min), yielding a cytoplasmic fraction, which was also stored on ice. Purified Hmp was oxidized) was taken to be 61.4 mM.

Experimental Procedures. Assays of enzyme and protein assays were used to determine the purity of periplasmic and cytoplasmic bacterial extracts were determined spectrophotometrically (38) except that an SDB-4 dual-wavelength scanning spectrophotometer was used (University of Pennsylvania Biomedical Instrumentation Group, and Current Designs Inc., Philadelphia, PA) (39). Dithionite-reduced minus persulfate-oxidized difference spectra were computed. Spectral data were analyzed and plotted using Soft SDB (Current Designs Inc.) and CA-Cricket Graph III software. The absorbance coefficient (ε_{422-413}) for cytochrome c_{552} (reduced minus oxidized) was taken to be 61.4 mM^{-1} cm^{-1}, deduced from the data of Fujita (40).

Evolution of NO from Wild-type E. coli—To measure N2O and NO, Ji and Hollocher (6, 13) used gas chromatography/mass spectrometry of headspace gases. In the present work, NO ev-
lution and \(O_2\) consumption were measured in solution using specific electrodes (21). Addition of formate to cells harvested from anaerobic, nitrate-supplemented cultures resulted in rapid \(O_2\) consumption in the closed reaction vessel. After reaching anoxia (Fig. 1A), the \(O_2\) concentration remained undetectable for the duration of the experiment. The NO electrode traces in Fig. 1B show that wild-type \(E. coli\) strain AN2342 produced NO, and that the amount of NO produced increased with the concentration of NaNO2 added (Fig. 1C). Thus, 2.5 mM NO2/H\(H_2O_2\) generated 10 nmol of NO/mg of protein and 5 mM NO2/H\(H_2O_2\) elicited 31 nmol NO/mg of protein. Even at high NO2/H\(H_2O_2\) concentrations (25 mM), NO evolution did not exceed 44 nmol of NO/mg of protein. To verify the selectivity of the electrode response, we used the imidazolineoxyl N-oxide, carboxy-PTIO, a stable radical compound that reacts stoichiometrically with NO to form either nitrate or nitrite and imidazolineoxyls in neutral solution (41). Addition of carboxy-PTIO to the electrode chamber rapidly decreased the electrode response in a dose-dependent manner (Fig. 1B), confirming that cells were producing NO. However, higher concentrations of carboxy-PTIO were required for diminution of the NO signal than would have been anticipated from the equistoichiometric reaction of carboxy-PTIO and the NO concentration determined by calibration of the electrode (see, for example, trace c, Fig. 1B). This might be due to loss of carboxy-PTIO owing to the lipophilicity and incorporation into bacterial membranes. Addition to the reaction chamber of deoxymyoglobin also quenched the NO signal (not shown). Cells cultured aerobically in the presence or absence of 100 mM KNO3 failed to produce NO (results not shown).

**Evolution of NO from Nitrite Reductase Mutants**—Previous work on NO evolution by \(E. coli\) has suggested that a nitrate reductase is responsible for the production of NO. Ji and Holllocher (6, 11, 13) concluded that NO production by \(E. coli\) is due to the reduction of nitrite to NO as a secondary activity of the membrane-bound respiratory nitrate reductase. Nitrosation reactions have also been genetically and biochemically linked to nitrate reductase genes (42–44). Smith (45) and Ralt et al. (44) correlated nitrous oxide production with nitrate reductase activity in \(E. coli\). However, Hutchings et al. (12) showed that the nitrate reductases of \(E. coli\) (all of which are molybdoenzymes) are not directly responsible for NO production from nitrite. Activation of NNR (an NO-responsive transcription factor of \(P. denitrificans\)) was abolished in an \(E. coli\) mobAB::Km mutant, defective in Mo cofactor biosynthesis, when nitrate, but not nitrite, was provided. The ability of certain nitrite reductases to reduce nitrite to NO (3) makes them strong candidates for the NO evolution observed here and by Hutchings et al. (12), particularly because \(E. coli\) lacks NO synthase.

\(E. coli\) possesses two nitrite reductases, namely a periplas-
mic cytochrome c enzyme (Nrf) that generates predominantly ammonium ion, and a cytoplasmic siroheme-dependent reductase (Nir) (46, 47). Therefore, we compared the NO evolution capacity of nir, nir nrf, and wild-type strains. Both the nir mutant JCB387 and the wild-type AN2342 produced NO, and use of carboxy-PTIO confirmed the presence of NO in both cases (Fig. 2A). The level of NO accumulated by the nir mutant (6.6 nmol/mg of cell protein, mean of two measurements) was approximately a third of that produced (17 nmol/mg of protein) by the wild-type strain. However, the nir nrf double mutant produced no detectable NO (Fig. 2A). The nrfA gene, implicated in NO production by this result, encodes a 52-kDa periplasmic pentaheme cytochrome c552, which acts as a nitrite reductase in association with the NrfB cytochrome c as redox partner. Therefore, we tested for the presence of cytochrome c in periplasmic and cytoplasmic extracts of the above strains. Dithionite reduced minus persulfate-oxidized spectra (Fig. 2B) revealed the presence of cytochrome c552 with a y-peak at 422 nm in both the wild-type strain AN2342 and the nir mutant JCB387 (0.10 and 0.11 nmol of cytochrome c552 per mg of protein, respectively). Weaker signals at 552 nm are due to the y-band but were not used for quantification. The NrfA protein has absorbance maxima in the reduced state (absolute spectrum) at 420.5 (y), 523.5 (beta), and 552 nm (alpha) (48). Purified NrfB protein, in its reduced state, has a sharp absorbance peak at 551 nm. Unlike NrfA, however, NrfB has no charge transfer band at approximately 630 nm. However, cytochrome c552 was undetectable in the nir nrf double mutant. As expected, cytoplasmic fractions (not shown) from all three strains showed no detectable levels of cytochrome c552 but the presence of b-type cytochrome(s) (not shown).

Effects of an hmp Mutation on NO Production—Hmp plays a key role in protecting cells against nitrosative stress by catalysis of either the oxygen-dependent formation of nitrate ion or the anaerobic reduction of NO to nitrous oxide (15). We hypothesized that an hmp mutant would produce significantly higher levels of NO compared with an isogenic wild-type strain, due to an inability to detoxify any NO formed. However, the results obtained were quite the opposite, as shown in Fig. 2C. The wild-type strain VJS676 produced NO (35 nmol of NO/mg of protein) at levels higher than wild-type strain AN2342 (23 nmol of NO/mg of protein; both means of two values). The hmp mutant strain RKP4545 produced no detectable NO, regardless of growth conditions (0–100 mM KNO3 in the medium) and the concentration of NaNO2 (in the range 2.5–25 mM) added to the electrode chamber. Given the demonstration (Fig. 2B) that nrf mutants lacking periplasmic cytochrome c552 were unable to generate NO from nitrite, we assayed cytochrome c552 in periplasmic and cyto-

---

31588

NO Production by E. coli

![Diagram](http://www.jbc.org/)

**Fig. 4.** An hmp mutant expressing Fnr* displays enhanced growth on nitrate and generates NO. A, cultures of VJS676 (wild-type, circles), RKP4545 (hmp, squares), and RKP2825 (hmp fur*, triangles) were grown anaerobically in the presence (100 mM KNO3, closed symbols) or absence (open symbols) of KNO3. Similar results were obtained in two experiments. B, measurements of NO production of washed cell suspensions of strains VJS676 (wild-type, top), RKP2825 (hmp fur*, middle), and RKP4545 (hmp, bottom) were made as described under “Experimental Procedures.” Cultures were grown anaerobically in the presence of 100 mM KNO3 and NaNO2 (25 mM) was added to washed cells. The results were similar in two experiments. The vertical bar corresponds to 68 μM NO (top) and 93 μM NO (middle). The horizontal bar corresponds to 29, 39, and 10 min, respectively. Respective protein concentrations were 0.6, 0.8, and 0.3 mg/ml.

---

2 D. Richardson, personal communication.
plasmic extracts of wild-type and hmp mutant strains. Dithionite reduced minus persulfate-oxidized spectra (Fig. 2D) revealed the presence of cytochrome c552 with peaks at 422 and 552 nm in the periplasm of the wild-type strain AN2342 (0.15 nmol of cytochrome c552 per mg of protein). The somewhat higher levels of cytochrome c in this strain correlate with higher levels of NO evolution (see above). However, cytochrome c552 was undetectable in the H9251-region of reduced minus oxidized spectra of the periplasm of the hmp mutant strain RKP4545, but a weak H9253-spectral signal at 422 nm was observed. As expected, cytoplasmic fractions (not shown) from both strains showed no detectable levels of cytochrome c552, but one or more b-type cytochromes were present. An Hmp-overproducing strain (RKP4717) also produced NO under these experimental conditions, but quadruplicate assays failed to show any significant differences from wild-type levels.

One explanation of the failure of an hmp mutant to generate NO might be that the flavohemoglobin Hmp itself is able to reduce nitrite ion to NO; indeed, Hmp possesses a broad spectrum of reductase activities (49). However, purified Hmp protein was unable to produce NO anoxically from nitrite. Briefly, 730 ng of purified Hmp protein was added to the electrode chamber, with 1.5 ml of PBS (pH 7.0). Oxygen levels were reduced to zero following addition of NADH, then 25 mM NO2 was added. In duplicate experiments, no NO evolution was observed (not shown).

Effects of an fnr Mutation on NO Production—The absence of NrfA or NrfB cytochromes in the hmp mutant prompted study of the link between Hmp, NO metabolism, and nitrite reductase. Recently we have demonstrated that the O2-responsive regulator Fnr, which represses hmp gene transcription, also senses NO (23). The [4Fe-4S]2+ cluster of Fnr reacts anaerobically with, and is inactivated by, NO. Active Fnr is a positive activator of the nrf operon, nirB, narG, and other genes involved in anaerobic nitrite and nitrate dissimilation (46). We therefore hypothesized that, because a mutant lacking Hmp is unable to detoxify NO, NO will inactivate Fnr, leading to a failure to up-regulate nrf (and other Fnr-regulated genes). If NrfA/NrfB are largely responsible for NO generation from nitrite, an fnr mutant should fail to produce NO. This hypothesis is supported by the results in Fig. 3A. Both wild-type strains used here showed similar levels of NO evolution (17 and 14 nmol of NO/mg of protein for strains AN2342 and RKP2178, respectively), whereas the fnr mutant (VJS5369) did not produce NO. The use of carboxy-PTIO again confirmed the presence of NO.

Mutational and NO Effects on Fnr Are Reflected in Anaerobic Growth Patterns—Anaerobic growth curves of the fnr mutant strain (VJS5369) and its corresponding wild-type strain (VJS5369) are shown in Fig. 3B. In the absence of added nitrate, growth of the fnr strain (RKP2178) proceeded rapidly after inoculation but after 10 h reached a saturation density of about 35 Klett units. Inclusion of nitrate substantially stimulated the growth rate in the first 10 h and then allowed growth to a significantly higher final cell yield (~58 Klett units). In contrast, the fnr mutant grew more slowly than the wild-type
in the first 10 h irrespective of the presence of nitrate. The inclusion of nitrate allowed a slower subsequent phase of growth, allowing the culture after 50 h to reach a final population density of about 56 Klett units.

To test further the hypothesis that an hmp mutant is defective in fnr-regulated gene function, anaerobic growth curves of wild-type (VJS676) and hmp mutant (RKP4545) strains were compared. Fig. 3C shows that the hmp mutant grew anaerobically in the absence of nitrate in a similar fashion to the wild-type. However, nitrate markedly stimulated growth of the wild-type strain but not of the hmp mutant. Wild-type strains VJS676 and AN2342 grew anaerobically in a similar fashion (results not shown). These growth data demonstrate that the fnr mutation adversely affects anaerobic growth and particularly anaerobic growth on nitrate. The data also reveal a previously undescribed phenotype of an hmp mutant, namely the inability of nitrate to stimulate anaerobic growth.

Fnr* Restores NO Evolution in an hmp Mutant—To further investigate the relationship between the E. coli flavohemoglobin and Fnr, an hmp strain carrying fnr* on a plasmid (50, 51) was constructed (RKP2825). It was hypothesized that this hmp fnr* strain would evolve NO, because the dimeric Fnr* protein is insensitive to NO inactivation even though the strain lacks a functional flavohemoglobin with which to detoxify NO. Under anoxic conditions, nitrate enhanced growth of the wild-type strain but not of the hmp mutant. Wild-type strains VJS676 and AN2342 grew anaerobically in a similar fashion (results not shown). These growth data demonstrate that the fnr mutation adversely affects anaerobic growth and particularly anaerobic growth on nitrate. The data also reveal a previously undescribed phenotype of an hmp mutant, namely the inability of nitrate to stimulate anaerobic growth.

Is NO Produced in a narG Mutant Strain?—In an attempt to clarify the role of the membrane-bound nitrate reductase in the production of NO, we tested the NO-producing ability of a narG mutant. The narG gene encodes the α-subunit of the membrane-associated respiratory nitrate reductase. NO was not produced by the narG mutant strain VJS789 when grown anaerobically in the presence of nitrate but was produced by wild-type strain AN2342 (results not shown). In the absence of nitrate in the growth medium, both the wild-type and the narG mutant strain failed to produce NO (results not shown). In view of the recent demonstration that nitrate reductase is not required for NNR activation (12), it is possible that the failure to detect NO evolution from the narG mutant is due to the lack of nitrite formed from Nar activity during growth.

NO Production by a cydD Mutant—Cytochrome bd is a terminal quinol oxidase (46), which appears to have additional physiological roles that are not readily explicable by its oxidase function (52). Hubbard et al. (26) observed a decrease in the absorbance maximum (630 nm) of reduced cytochrome d in membrane particles upon the addition of nitrate ions. Nitrite, trioxodinitrate, and NO also caused qualitatively similar, but faster, changes in the spectrum of cytochrome d. Nitrate gave a slower reaction rate, possibly due to a rate-determining reduction of nitrate to nitrite catalyzed by a nitrate reductase. It was concluded (26, 27) that this spectral shift was due to the presence of a cytochrome d-nitrosyl complex. Haddock et al. (53) also observed a shift in the spectrum of cytochrome d following

![Diagram: Scheme describing NO evolution and consumption in E. coli, and the proposed roles of Fnr and Hmp. For details, see text. The [4Fe-4S]^{2+} and nitrosylated clusters of Fnr are represented by cubes and an octagon, respectively.](https://example.com/diagram.png)
anaerobic growth on nitrate. The above evidence led us to investigate the role of cytochrome bd in NO evolution in *E. coli*. Fig. 5 shows that NO is evolved by a cycD mutant at levels (11 and 28 nmol of NO/mg of protein at 12.5 and 25 mM NO$_3^-$, respectively) approximately one-third less than its wild-type. Thus cytochrome bd is not essential for NO production.

**DISCUSSION**

Our results demonstrate NO$_3^-$-dependent NO evolution in *E. coli* although at lower levels than those measured before (13). These differences could be due to the different strains used or techniques employed in the measurement of NO.

The mechanism of NO formation during denitrification are reasonably well understood as a result of high resolution x-ray structures of both classes of nitrite reductases in which the substrate (NO$_2^-$) or product (NO) are bound at the active site (see (5)). In *E. coli* however, both nitrite reductases were thought to be assimilatory, producing ammonium ion, not NO, until now (47).

In our work, NO generation was not detectable in an *nrf* mutant, nor in *nrf* strains in which the *nrf*-encoded nitrite reductase is not expressed or synthesized at spectrally detectable levels, as a consequence of *hmp* or *fnr* mutations. Expression of the *NrfA* and *NirB* operons is elevated during anaerobic growth by *Fnr* (29, 54). Wang and Gunsalus (55) concluded that *nrfA* operon expression is induced only when nitrite concentrations are low, whereas *NirB* seems to be optimally synthesized only when nitrate or nitrite are in excess of the cell's capacity to consume them.

The physiological significance of NO generation by Fnre-mains to be determined. However, the present mutant-based analysis suggests that other NO-generating mechanisms that have been proposed are insignificant when cells are grown anaerobically with NO$_3^-$. These include the chemical reduction of nitrite by ferrous ion (56) or by formate (57). Zumft (58) concluded that *nrfA* operon expression is induced only when nitrite concentrations are low, whereas *NirB* seems to be optimally synthesized only when nitrate or nitrite are in excess of the cell’s capacity to consume them.

The physiological significance of NO generation by Nrf remains to be determined. However, the present mutant-based analysis suggests that other NO-generating mechanisms that have been proposed are insignificant when cells are grown anaerobically with NO$_3^-$. These include the chemical reduction of nitrite by ferrous ion (56) or by formate (57). Zumft (58) suggested that non-enzymatic transformations could be responsible for NO formation in non-denitrifiers, as demonstrated in humans; inorganic nitrite is chemically reduced to NO under acidic, reducing conditions (59). “Chemodenitrification” has also been suggested as an NO-producing mechanism (60), involving the decomposition of hydroxylamine into NO and N$_2$O (58). However, the reducing, acidic conditions required were not met in our electrode chamber experiments.

An *hmp* mutation prevented NO formation, presumably a consequence of the failure of Hmp to remove the NO that accumulates during growth with nitrate. However, under the anoxic conditions employed here, it is improbable that the “oxgenase” (19) or denitrosylase (61) activity of Hmp in which NO is converted to nitrate could operate, particularly because the apparent *Km* of this reaction for O$_2$ is relatively high (50–90 μM) (16, 21). Recent work by Gardner and Gardner (62) suggests that, although Hmp is highly efficient as an oxygen-dependent NO-detoxifying enzyme, its role in anaerobic NO metabolism and detoxification is minimal and a flavorubredoxin is proposed to possess NO-scavenging activity during anaerobic growth. However, it should be noted that, although *hmp* transcription is not significantly up-regulated anaerobically compared with aerobically, the presence of nitrite, and especially nitrite and NO, which will be formed under the growth conditions used here, dramatically up-regulate *hmp* transcription (18). This increase may provide sufficient NO-detoxifying activity to protect Fnr from inactivation. Nrf is periplasmically located, so that NO damage to Fnr and intracellular targets will require facile NO diffusion to the cytoplasm. Thus, membrane-associated NO-detoxifying activity would be particularly effective in NO removal, and there is evidence for the presence of Hmp apoprotein in the *E. coli* periplasm (63).

A model of the fate of nitric oxide in *E. coli*, is shown in Fig. 6. Fnrs senses pathophysiological levels of NO (5–10 μM) in *vivo*, and NO inactivates Fnr (23). Thus, one consequence of NO accumulation within *E. coli* would be loss of a major mechanism (via Fnr) for up-regulation of nitrate and nitrite reductases. During anaerobic growth, the potentially harmful accumulation of NO from the combined activities of nitrate and nitrite reductases is prevented by the combined NO-detoxifying activities of Hmp and flavorubredoxin, both of which are up-regulated under such conditions (18, 62). However, mutation of *hmp* alone appears sufficient for NO accumulation, inactivation of Fnr, and consequent down-regulation of *nrf* operons, and other genes required for anaerobic growth. Failure to form nitrate reductase is shown in the present work by the lack of growth stimulation of an *hmp* mutant by nitrate (Fig. 3C), whereas loss of periplasmic nitrite reductase in an *hmp* mutant is reflected in the spectral analysis. Although lacking a functional flavohemoglobin, the *hmp fnr* strain generated NO (Fig. 4B), demonstrating the role of Fnr inactivation. Strain RKP4545 (*hmp*) displayed poorer anaerobic growth in the presence of nitrate (Fig. 3C) than did the *fnr* mutant (Fig. 3B). Although Fnr inactivation has global effects on anaerobic growth capability, the consequences of mutation of *hmp* appear more severe, because of failure to detoxify NO that arises from Fnr-independent routes and multiple sites of NO toxicity.

Although the reaction of nitrite with flavohemoglobin might be anticipated to be similar to the reaction of nitrite/nitrous acid with human deoxyhemoglobin (64), we could find no evidence that purified Hmp forms NO from nitrite under anaerobic conditions. Nitrate reductase-dependent mutagenesis in *E. coli* was observed in LB medium containing no added nitrate or nitrite (65). The requirement for hypoxia for maximum mutagenesis by nitrite suggests that hypoxia may induce an enzyme that generates NO from nitrate as proposed here for nitrate reductase(s).

Finally, the formation of free NO in cells growing under conditions of NO$_3^-$ respiration has major implications for energy metabolism in facultative bacteria. Because NO is a potent inhibitor of both terminal oxidases in *E. coli* (66), formation of nitrite by anaerobic respiratory enzymes may inhibit oxidase activity, thereby augmenting the shut-down of aerobic metabolism activated primarily by regulation of gene expression.

**Acknowledgments**—We thank J. Cole and V. Stewart for generously providing strains; J. Green for suggesting the *fnr* experiment and donating the *fnr* plasmid; J. Cole, M. N. Hughes, D. Lloyd, C. Mills, D. Richardson, and G. Wu for useful advice and discussions; and M. Johnson for technical support and assistance in compiling figures.

**REFERENCES**

1. Stuehr, D. J. (1999) *Biochim. Biophys. Acta* 1411, 217–230
2. Elffering, S. L., Sarkela, T. M., and Giuli, C. (2002) *J. Biol. Chem.* 277, 38079–38086
3. Cattrazza, F. (1999) *Biochim. Biophys. Acta* 1411, 231–249
4. Zumft, W. G. (1997) *Microbiol. Mol. Biol. Rev.* 61, 253–264
5. Watmough, N. J., Butland, G., Cheesman, M. R., Mair, J. W. B., Richardson, D. J., and Spiro, S. (1999) *Biochim. Biophys. Acta* 1411, 456–470
6. Ji, X.-B., and Hollocher, T. C. (1988) *Appl. Environ. Microbiol.* 54, 1791–1794
7. Anderson, I. C., and Levine, J. S. (1986) *Appl. Environ. Microbiol.* 51, 938–945
8. Kalkowski, I., and Conrad, R. (1989) *FEMS Microbiol. Lett.* 62, 107–112
9. Bu, T. Y., and Knowles, R. (2000) *Appl. Environ. Microbiol.* 66, 3891–3897
10. Mallick, N., Mohn, F. H., and Soeder, C. J. (2000) *J. Plant. Physiol.* 157, 40–46
11. Xu, X. B., and Hollocher, T. C. (1998) *Biochem. Arch.* 5, 81–86
12. Hutchings, M. I., Shearer, N., Wastell, S., van Spanning, R. J. M., and Spiro, S. (2000) *J. Bacteriol.* 182, 6434–6439
13. Ji, X.-B., and Hollocher, T. C. (1988) *Biochem. Biophys. Res. Commun.* 157, 106–108
14. Zhang, X., and Brederick, M. (2000) *Mod. Asp. Immunol.* 1, 160–165
15. Poole, R. K., and Hughes, M. N. (2000) *Mod. Microbiol.* 36, 773–783
16. Gardner, P. R., Martin, L. A., Hall, D., and Gardner, A. M. (2000) *J. Biol. Chem.* 275, 31581–31587
17. Pfoot, S. R., Leach, E. R., Mair, J. W. B., Cole, J. A., and Richardson, D. J.
