Respiratory Detoxification of Nitric Oxide by the Cytochrome c Nitrite Reductase of Escherichia coli*

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Nitric oxide is a key element in host defense against invasive pathogens. The periplasmic cytochrome c nitrite reductase (NrfA) of Escherichia coli catalyzes the respiratory reduction of nitrite, but in vitro studies have shown that it can also reduce nitric oxide. The physiological significance of the latter reaction in vivo has never been assessed. In this study the reduction of nitric oxide by Escherichia coli was measured in strains active or deficient in periplasmic nitrite reduction. NrfA cells, harvested from cultures grown anaerobically, possessed a nitric-oxide reductase activity with physiological electron donation of 60 nmol min⁻¹·mg dry wt⁻¹, and an in vivo turnover number of NrfA of 390 NO⁻ s⁻¹ was calculated. Nitric-oxide reductase activity could not be detected in Nrf⁻ strains. Comparison of the anaerobic growth of Nrf⁺ and Nrf⁻ strains revealed a higher sensitivity to nitric oxide in the Nrf⁻ strains. A higher sensitivity to the nitrosating agent S-nitroso-N-acetyl penicillamine (SNAP) was also observed in agar plate disk-diffusion assays. Oxygen respiration by E. coli was also more sensitive to nitric oxide in the Nrf⁻ strains compared with the Nrf⁺ parent strain. The results demonstrate that active periplasmic cytochrome c nitrite reductase can confer the capacity for nitric oxide reduction and detoxification on E. coli. Genomic analysis of many pathogenic enteric bacteria reveals the presence of nrf genes. The present study raises the possibility that this reflects an important role for the cytochrome c nitrite reductase in nitric oxide management in oxygen-limited environments.

Nitrogen monoxide (nitric oxide or NO⁻) has long been recognized as a free intermediate in the denitrification reactions of the nitrogen cycle, in which bacteria reduce nitrate or nitrite to gaseous nitrogen oxides and dinitrogen (1, 2). The bacteria that participate in denitrification can utilize the NO⁻ free radical as a substrate for an energy-conserving respiratory electron transport pathway that terminates in an integral membrane nitric-oxide reductase that is a member of the heme-copper oxidase superfamily (3, 4). This enzyme is also found in many non-denitrifying bacteria, including the phototroph Rhodobacter capsulatus (5), and may play a role in detoxifying NO⁻ produced by other bacteria in the organism’s environment (2, 6). In the last decade it has also become clear that NO⁻ is a key component of the host defense response to invasion by pathogenic bacteria (7). For example, NO⁻ is produced by macrophages through the activity of inducible nitric-oxide synthase, which converts L-arginine to cytotoxic NO⁻. The NO⁻ can, in turn, form a number of other reactive nitrogen and oxygen species, including NO⁺, NO⁻, N₂O₃, and ONOO⁻, all of which can have bactericidal or bacteriostatic effects on phagocytosed organisms (7–9).

Studies indicate that many pathogenic bacteria may have one or more means of removing NO⁻ from their environment. For example, in oxic environments the denitrosylase reaction of flavohemoglobin, which can turn over at 600–700 NO⁻ s⁻¹, may play a role in NO⁻ resistance in Escherichia coli and Salmonella typhimurium (9–13). The anaerobic reduction of NO⁻ to N₂O by E. coli flavohemoglobin has also been demonstrated (11). However, this reaction is rather slow compared with the aerobic reaction. Its importance in anaerobic NO⁻ detoxification in E. coli has recently been questioned, and a NO⁻-inducible cytoplasmic NADH-dependent flavoredoxin has been implicated in NO⁻ reduction (15, 16).

Respiratory detoxification of NO⁻ by pathogens may also be important. For example, analysis of the genome sequences of some pathogens, such as Neisseria meningitidis, has revealed genes for a respiratory NO⁻ reductase of the heme-copper oxidase family that have been well characterized in denitrifying bacteria (6). In the latter bacteria, these enzymes can turnover NO⁻ at around 50–100 s⁻¹ (3, 4), and so they could potentially provide a pathogen with an effective means of rapidly removing NO⁻ in oxygen-limited environments. The site of NO⁻ reduction is likely to be toward the periplasmic face of the membrane, which would help to detoxify the reactive radical before it enters the cell.

Respiratory nitric-oxide reductases are absent from many enteric pathogens, such as Escherichia and Salmonella species. However, analysis of the genome sequences of many pathogenic enteric bacteria has revealed the presence of a nrfA gene encoding a pentaheme periplasmic cytochrome c nitrite reductase (17). This respiratory enzyme catalyzes the six-electron reduction of nitrite to ammonia and is normally expressed under anoxic or micro-oxic growth conditions in the presence of nitrate and nitrite (18–20). The x-ray crystal structures of NrfA from E. coli, Wolinella succinogenes, and Sulfospirillum deleyianum have recently been solved and are leading to a

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1 The abbreviations used are: NrfA, cytochrome c nitrite reductase; SNAP, S-nitroso-N-acetyl penicillamine; SNOG, S-nitroso glutathione; MS, minimal salt.
greater understanding of the enzymatic mechanism for nitrite reduction (21–23). Perhaps significantly, it has been recognized for a number of years that NrfA can also catalyze the five-electron reduction of NO\(^-\) to NH\(_4^+\), with turnover rates estimated at between 30 and 1000 s\(^{-1}\) (24). The physiological significance of this reaction has never been investigated, yet it is clearly possible that in oxygen-limited environments NrfA contributes to the removal of potentially cytotoxic NO\(^-\). In the case of pathogenic enteric bacteria, it is recognized that low oxygen tensions are encountered during infection. It is notable that S. typhi mutants deficient in anaerobic respiration have impaired ability to replicate within epithelial cells (25). In other enteric bacteria, strains of E. coli deficient in NrfA activity (26) were assessed for NO\(^-\) reduction with physiological electron donors in intact cells of E. coli and have shown that growth of mutants deficient in NrfA activity is more sensitive than that of the parent strain to NO\(^-\). This demonstrates, for the first time, a respiratory route for anoxic NO\(^-\) management in E. coli. This route of NO\(^-\) management may be widespread among other enteric bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The strains used were E. coli JCB387 (ΔnirB), E. coli JCB352\(^{tr}\) (ΔnirB nrf::mud1Aplac), and E. coli JCB362\(^{tr}\) (ΔnirB nrf::mud1Aplac) (26). Aerobic cultures were grown either in LB medium (10 g of tryptone, 10 g of NaCl, 5 g of yeast extract per liter, pH 7.5) or on LB agar solidified with 1.5% w/v agar. Aerobic 20 ml cultures were grown overnight in 150 ml conical flasks shaken at 200 rpm. Anaerobic cultures were grown in minimal salts (MS) medium (27), supplemented with 1% LB, 10 μM selenite, and 10 μM molybdate. The main carbon source was 0.4% (w/v) glycerol, and the terminal electron acceptors were 20 mM sodium nitrate and 40 mM sodium fumarate. For anaerobic growth on solid medium, 1.5% w/v agar was added to the MS media. To ensure there was no oxygen interference, liquid cultures were grown with no aeration in screw-cap bottles that were filled to the top; plates were secured in an anaerobic jar with an oxygen indicator and an anaerobic gas pouch that releases hydrogen and carbon dioxide (purchased from Oxoid). Ampicillin (100 μg ml\(^{-1}\)) was added where appropriate. Bacteria were grown at 37 °C. Solid agar suspensions of E. coli were made by mixing 1 part of an overnight liquid culture with 9 parts melted 0.3% agar medium instead of 1.5% agar medium.

**Measurement of Nitric-oxide Reductase Activity**—E. coli were grown anaerobically overnight, and 100 ml of each culture was centrifuged at 6000 rpm and resuspended in 10 ml of the spent medium. A Clarke-type oxygen electrode was polarized to detect nitric oxide, and 3 ml of the concentrated cell culture was put into the electrode chamber. The cells were allowed to consume the oxygen present in the chamber, and a...
FIG. 3. The effect of nitric oxide on anaerobic growth of \textit{E. coli} in semisolid medium in Hungate tubes. Nitric oxide gas was injected into the headspace of Hungate tubes containing semisolid agar suspensions of the test strain. A, JCB362\textsuperscript{TR} (nrf\textsuperscript{+}) control tube containing no nitric oxide (left); JCB362\textsuperscript{TR} with 50 \(\mu\)l of nitric oxide gas injected into the headspace (right). B, JCB387 (nrf\textsuperscript{−}) with 50 \(\mu\)l of NO\textsubscript{2} gas injected into the headspace (left). JCB362\textsuperscript{TR} with 50 \(\mu\)l of nitric oxide gas injected into the headspace (right).

FIG. 4. The sensitivity of \textit{E. coli} strains JCB387 and JCB362\textsuperscript{TR} to SNAP. Paper disks soaked in a solution of SNAP were placed on plates containing agar suspensions of the test strain. A, JCB387 (Nrf\textsuperscript{+} parent strain) with SNAP-soaked disk (left); JCB387 with control disk containing no SNAP (right). B, JCB362\textsuperscript{TR} (Nrf\textsuperscript{−}) with SNAP-soaked disk (left); JCB362\textsuperscript{TR} with control disk containing no SNAP (right).

known quantity of a saturated NO solution (2 mm) was added to the chamber in order to obtain a nitric oxide signal. The rate at which the cells consumed the nitric oxide was observed. During control experiments in which no \textit{E. coli} cells were present, glucose, glucose oxidase, and catalase were added to the chamber to consume the oxygen present. The electrode chamber was filled with 5 ml of 50 mm HEPES, 50 mm KCl (pH 7.1). Glucose (to 16 mm), glucose oxidase (to 4 units ml\(^{-1}\)), and catalase (to 20–25 units ml\(^{-1}\)) were added. Once the oxygen had been consumed, known quantities of a saturated NO solution were added as described above.

\textit{Disk Diffusion Susceptibility Assays}—Agar suspensions of the \textit{E. coli} to be tested were poured onto plates, and 4-mm Whatman paper disks that had been soaked with 15 \(\mu\)l of a 250 mm solution of the NO-releasing compound SNAP were placed carefully on the center of the plates. Plates were then incubated anaerobically overnight.

\textit{NO Resistance Tests in Semisolid Agar}—Agar suspensions of the \textit{E. coli} cells to be tested were poured into Hungate tubes and allowed to set. Known quantities of NO gas were then injected into the headspace of the tubes using a gas-tight Hamilton syringe. The tubes were then incubated overnight.

\textit{Cell Fractionation}—Bacteria were grown anaerobically overnight and harvested by centrifugation; the supernatant was discarded. The pellet was resuspended in 10% of the original culture volume of spheroplast buffer (500 mm sucrose, 100 mm Tris, and 2 mm EDTA, pH 8.0), and lysozyme was added to a concentration of 2 mg/ml. After 30 min at 37 \(^{\circ}\)C, the sample was centrifuged for 30 min at 14,000 rpm at 4 \(^{\circ}\)C. The supernatant periplasm was retained for analysis.

\textit{Gel Electrophoresis}—Proteins present in the soluble \textit{E. coli} extract were separated on SDS/15% PAGE. The gel was then stained for covalently bound heme (28).

\textit{Nitrite Reductase Assays}—Cultures (30 ml) that had been grown either aerobically or anaerobically were harvested by centrifugation at 4 \(^{\circ}\)C for 15 min at 10,000 rpm in a Sigma desktop centrifuge. The bacterial pellet was washed twice by resuspension in 30 ml of ice-cold LB that had been precooled to prevent heat shock; centrifugation was as above. The washed bacteria were resuspended in 3 ml of 20 mm Hepes buffer, pH 8.0; 2.5 ml of this suspension was transferred to a 3-ml glass cuvette containing 25 \(\mu\)l of 100 mm methyl viologen and a magnetic flea. The top of the cuvette was sealed with a Suba-Seal septum into which two stainless steel needles had been inserted. The contents of the cuvette and the headspace were sparged with oxygen-free nitrogen gas for 15 min. The methyl viologen was reduced with a freshly prepared solution of 100 mm sodium dithionite dissolved in Hepes buffer that had been degassed with oxygen-free nitrogen for 20 min. When an optical density of 600 nm due to reduced methyl viologen was constant, the reaction was started by the addition of about 3 \(\mu\)l of 1 m sodium nitrite; the rate of oxidation of methyl viologen was determined in an Aminco spectrophotometer.

\textit{Measurement of Oxygen Consumption}—Cells were grown anaerobically overnight. 3 ml of cells were put into the chamber of a Clarke-type oxygen electrode, and the rate of oxygen consumption was observed. Once a steady rate of oxygen consumption had been established, a known quantity of an NO–saturated solution (2 mm) was added to the chamber. Any resulting inhibition of oxygen consumption was observed.

\textit{Growth Curves}—Anaerobic growth curves were carried out in sterilized 3 ml glass cuvettes. 50 ml of MS medium was inoculated with 0.5 ml of an overnight culture, and 3 ml aliquots were put into the cuvettes and sealed with sterilized Suba-Seals. The contents of the cuvettes were then sparged with oxygen-free nitrogen gas for 15 min. The OD\textsubscript{600} of the cultures was taken every hour. Once the OD\textsubscript{600} of the experimental cultures had reached 0.10, 50 \(\mu\)l of NO\textsubscript{2} gas was injected into the cultures using a sterilized gas-tight Hamilton syringe.

\textbf{RESULTS}

\textit{Measurement of NO\textsubscript{2} Reduction in \textit{E. coli}}—To assess whether NrfA can make a contribution to NO\textsubscript{2} reduction in \textit{E. coli}, NO\textsubscript{2} consumption was assessed in two nrf\textsuperscript{−}:mud1Aplac insertion mutants, JCB352\textsuperscript{TR} and JCB362\textsuperscript{TR}, and the parent strain JCB387. These strains are all deficient in the cytoplasmic siroheme nitrite reductase, NirA. Their use thereby prevents any possible complication from contributions that this enzyme might make to nitrite and nitric oxide reduction. The Nrf\textsuperscript{−} strains could not grow anaerobically on the non-fermentable carbon source glycerol with NO\textsubscript{2} present as sole electron acceptor. Consequently all three strains were grown anaerobically with glycerol as the carbon and energy source and with nitrate and fumarate present as respiratory electron acceptors. The growth rate and final optical density of the Nrf\textsuperscript{−} strains were both slightly less than the parent strain under these conditions (Fig. 1). The expression of active NrfA was assessed using reduced methyl viologen (MV\textsuperscript{−}) as a non-physiological electron donor. A MV\textsuperscript{−}-dependent nitrite reductase activity of
Paracoccus denitrificans reductase, such as recorded in other bacteria that can express the respiratory NO \( \text{NO}_2^- \) is also comparable with anaerobic NO \( \text{NO}_2^- \) latus (5). The time courses of NO about 10 \( \text{NO}_2^- \) \( \mu \text{M} \) was absent. This was possibly a consequence of competitively on glycerol/nitrate/fumarate medium were assessed for NO' reduction activity. Cells collected from late exponential-phase cultures were harvested, washed, and resuspended in glycerol-supplemented growth medium without electron acceptors. NO' consumption was monitored using a Clarke-type electrode under anaerobic conditions. The JCB387 parent strain reduced NO' at a maximum rate of 300 nmol of NO' per mg of protein min \(^{-1} \). This was comparable with the rates of nitrate, nitrite (not shown), and oxygen (Fig. 5) reduction by \( E. \text{coli} \). It is also comparable with anaerobic NO' respiration rates recorded in other bacteria that can express the respiratory NO' reductase, such as \( \text{Paracoccus denitrificans} \) (29) and \( R. \text{capsulatus} \) (5). The time courses of NO' reduction were linear down to about 10 \( \mu \text{M} \) NO', which suggests that the bacterial cells have a low \( K_c \) (high affinity) for this substrate under these assay conditions. The NO' was not toxic to the JCB387 cells, because repeated pulses of NO' over a 3-h period were also consumed at a similar rate. Measurement of ammonium accumulation in the assay medium following NO' consumption revealed a nearest integer ratio of 1 mol of NO' consumed per 1 mol of \( \text{NH}_4^+ \) produced. Consumption of NO' was not observed in cells harvested from cultures grown under highly aerated conditions, which correlated with an absence of \( nrf \) expression under these culture conditions. The anaerobic consumption of NO' could not be detected when measured under comparable conditions in either of the two \( \text{Nrf}^- \) strains, JCB352\text{TR} and JCB362\text{TR} (Fig. 2). The slight downward drift of the electrode trace apparent in these experiments was also observed when cells were left out of the electrode chamber. In the parent strain JCB387, anaerobic NO' reduction was also observed in the presence of 5 \( \text{mm} \) nitrite or in spent culture medium that contained nitrite. Under these conditions NO' reduction was about 50\% slower than when nitrite was absent. This was possibly a consequence of competition for the active site of NrfA between NO' and NO_2^-.

100 nmol mg dry wt \(^{-1} \) min \(^{-1} \) was measured in JCB387, but no activity could be detected in either JCB352\text{TR} or JCB362\text{TR} (Fig. 1, inset).

Having established that active NrfA was present in JCB387 and absent in JCB352\text{TR} and JCB362\text{TR}, cultures grown anaerobically on glycerol/nitrate/fumarate medium were assessed for NO' reduction activity. Cells collected from late exponential-phase cultures were harvested, washed, and resuspended in glycerol-supplemented growth medium without electron acceptors. NO' consumption was monitored using a Clarke-type electrode under anaerobic conditions. The JCB387 parent strain reduced NO' at a maximum rate of 300 nmol of NO' per mg of protein min \(^{-1} \). This was comparable with the rates of nitrate, nitrite (not shown), and oxygen (Fig. 5) reduction by \( E. \text{coli} \). It is also comparable with anaerobic NO' respiration rates recorded in other bacteria that can express the respiratory NO' reductase, such as \( \text{Paracoccus denitrificans} \) (29) and \( R. \text{capsulatus} \) (5). The time courses of NO' reduction were linear down to about 10 \( \mu \text{M} \) NO', which suggests that the bacterial cells have a low \( K_c \) (high affinity) for this substrate under these assay conditions. The NO' was not toxic to the JCB387 cells, because repeated pulses of NO' over a 3-h period were also consumed at a similar rate. Measurement of ammonium accumulation in the assay medium following NO' consumption revealed a nearest integer ratio of 1 mol of NO' consumed per 1 mol of \( \text{NH}_4^+ \) produced. Consumption of NO' was not observed in cells harvested from cultures grown under highly aerated conditions, which correlated with an absence of \( nrf \) expression under these culture conditions. The anaerobic consumption of NO' could not be detected when measured under comparable conditions in either of the two \( \text{Nrf}^- \) strains, JCB352\text{TR} and JCB362\text{TR} (Fig. 2). The slight downward drift of the electrode trace apparent in these experiments was also observed when cells were left out of the electrode chamber. In the parent strain JCB387, anaerobic NO' reduction was also observed in the presence of 5 \( \text{mm} \) nitrite or in spent culture medium that contained nitrite. Under these conditions NO' reduction was about 50\% slower than when nitrite was absent. This was possibly a consequence of competition for the active site of NrfA between NO' and NO_2^-.

The results with the nitric oxide electrode clearly demonstrated that the Nrf-dependent respiratory electron transport system of \( E. \text{coli} \) could account for significant rates of NO' reduction in anaerobic intact cell suspensions. Previous in vitro measurements of NO' reduction by NrfA from a number of bacterial sources have yielded rates that lie in the range of 30–1000 NO' s \(^{-1} \) (24). The large variation probably arises, at least in part, from the use of different non-physiological electron donors (e.g. dithionite or ascorbate). The present study has assessed NO' consumption by NrfA in the intact cells with electron donation through a physiologically relevant route. The in situ turnover number was estimated by quantifying NrfA in cells by comparing the intensity of the 52-kDa NrfA hematin-staining band in periplasmic fractions of JCB387 to that of purified NrfA standards (Fig. 2C). This yielded a value of 12.8 pmol of NrfA (mg of protein \(^{-1} \) and allows the turnover rate for NO' in the intact cell to be estimated at 390 s \(^{-1} \), which is within the range reported from the in vitro enzymatic assays.

Resistance of \( E. \text{coli} \) to NO' during Anaerobic Growth in Liquid and Semisolid Cultures—To assess whether NrfA can confer NO' resistance during anaerobic growth of \( E. \text{coli} \), NO' was added to actively growing cultures during the early exponential phase of growth (Fig. 1B). Anaerobic growth of the \( \text{Nrf}^- \) strains, JCB352\text{TR} and JCB362\text{TR}, was more sensitive to NO' than growth of the parent strain, JCB387. Thus, addition of less than 50 \( \mu \text{mol} \text{ml}^{-1} \) NO' did not inhibit growth of any of the strains (data not shown). However, addition of 150 \( \mu \text{mol} \text{ml}^{-1} \) NO' completely attenuated growth of the JCB352\text{TR} and JCB362\text{TR} but had no effect on the growth kinetics of JCB387 (Fig. 1B). At higher NO' (200 nmol of NO' ml \(^{-1} \)), a transient 4-h growth inhibition of JCB387 was observed, but the culture recovered fully and thereafter exhibited normal growth kinetics (data not shown). Sensitivity to NO' was also assessed during growth in Hungate tubes in semisolid agar. For JCB352\text{TR} and JCB362\text{TR}, clear zones were observed at the tops of the Hungate tubes in which 100 nmol of NO' had been injected into the headspace (Fig. 3). By contrast, in the absence of NO' the bacteria colonized the agar right up to the agar-gas interface. No clear zones were apparent in the presence of 100

**Fig. 5.** The effect of nitric oxide on oxygen respiration by \( E. \text{coli} \) strains JCB387, JCB352\text{TR}, and JCB362\text{TR}.

The full scale deflection was equivalent to 236 nmol ml \(^{-1} \) \( \text{O}_2 \). 30 nmol ml \(^{-1} \) nitric oxide was added where indicated.
nmol of NO\textsuperscript{−} in Hungate tubes containing JCB387. The clear zones observed with JCB352\textsuperscript{TR} and JCB362\textsuperscript{TR} could arise from either growth inhibition or bacterial taxis away from the chemotoxic NO\textsuperscript{−}. Either way, the experiment demonstrates that the Nrf\textsuperscript{−} strains are less able to manage NO\textsuperscript{−} than is a Nrf\textsuperscript{+} parent strain.

Disk diffusion sensitivity assays were used to compare the growth sensitivity of the Nrf mutants, strains JCB352\textsuperscript{TR} and JCB362\textsuperscript{TR}, and the parental strain, JCB387, to SNAP and SNOG during both aerobic and anaerobic growth. Under both conditions, the mutant strains were far more sensitive than the parent to SNAP (Fig. 4, only anaerobically incubated plates are shown). The ability of NrfA to protect the parental strain even on aerobically incubated plates indicated that at least parts of the bacterial colonies were sufficiently oxygen-depleted to allow Nrf activity to be expressed. This was confirmed by showing that bacteria scraped from these plates could catalyze nitrite reduction by methyl viologen. No zones of growth inhibition by SNOG were observed for any of the strains. This is consistent with a recent report that E. coli can metabolize and hence detoxify SNOG (30).

Oxygen Uptake Assays—NO\textsuperscript{−} can bind to metal centers in respiratory enzymes, including oxidases, resulting in inhibition (12, 31). The effect of NO\textsuperscript{−} on oxygen utilization was investigated by adding NO\textsuperscript{−} to cell suspensions respiring oxygen monitored by a Clarke-type electrode. All three strains respired oxygen at similar rates (120 nmol of O\textsubscript{2} min\textsuperscript{−1} mg dry wt\textsuperscript{−1}) (Fig. 5). Previous studies of NO\textsuperscript{−} inhibition of oxygen respiration have demonstrated that sensitivity is highest at low oxygen concentrations (12). Thus in the present experiments care was made to compare the effect of NO\textsuperscript{−} on oxygen respiration in JCB387, JCB352\textsuperscript{TR}, and JCB362\textsuperscript{TR} at similar oxygen concentrations, around 150–175 nmol ml\textsuperscript{−1} O\textsubscript{2} (63–74% air saturation). Addition of 30 nmol ml\textsuperscript{−1} NO\textsuperscript{−} to the JCB387 strain did not inhibit oxygen uptake (Fig. 5). However, the same quantity of NO\textsuperscript{−} added to either of the Nrf\textsuperscript{−} strains, JCB352\textsuperscript{TR} or JCB362\textsuperscript{TR}, strongly attenuated NO\textsuperscript{−} reduction (Fig. 5). Addition of NO\textsuperscript{−} over the range of 30 nmol ml\textsuperscript{−1} to 100 nmol ml\textsuperscript{−1} established that the period of inhibition was proportional to the amount of NO\textsuperscript{−} added (not shown). At concentrations greater than 100 nmol ml\textsuperscript{−1}, NO\textsuperscript{−} reversible inhibition of oxygen uptake by JCB387 was also observed, but the experiment clearly shows that oxygen respiration is more sensitive to NO\textsuperscript{−} addition in JCB352\textsuperscript{TR} or JCB362\textsuperscript{TR} than in JCB387.

DISCUSSION

Many enteric bacteria will encounter nitric oxide when living within a host or living outside a host. For example, enteric bacteria present in soils, sediments, and water systems will encounter NO\textsuperscript{−} generated by denitrifying and nitrifying bacteria. Enteric bacteria in the gut will encounter NO\textsuperscript{−} generated by denitrifying bacteria from gut nitrates. Pathogenic enteric bacteria infecting mammalian cells will encounter NO\textsuperscript{−} produced by the oxidation of l-arginine by the nitric-oxide synthase. Genome analysis of many enteric bacteria has revealed the presence of a nrfA homologue (17). The present study has raised the possibility that NrfA could play a role in protecting bacteria against the lethal effects of NO\textsuperscript{−}. nrfA expression is highest following exposure to anaerobic environments (18–20), and so it is likely that active NrfA will be present following passage through the gut where the organism will also be exposed to NO\textsuperscript{−}. Furthermore, the oxygen electrode experiments of this study have shown that expression of nrf following anaerobic growth still allows protection of oxygen respiration in oxic environments. Thus if bacteria phagocytosed by macrophages have active NrfA, this could consume NO\textsuperscript{−} produced by nitric-oxide synthase and thereby minimize the formation of reactive nitrogen species.

In the case of E. coli, other significant mechanisms of NO\textsuperscript{−} resistance have been reported, namely the aerobic and anaerobic reactions of flavohemoglobin (9–14). However, recent research has also revealed the presence of an important inducible system involved in the reduction and detoxification of NO in E. coli (15, 16). The system includes a transcription regulator controlling anaerobic NO\textsuperscript{−} reduction, a flavoborixedoxin with an NO\textsuperscript{−}-binding non-heme diiron center, and a NADH:flavorubiredoxin oxidoreductase. The NO\textsuperscript{−} reductase activity of the flavoborixedoxin efficiently removes NO\textsuperscript{−} in anaerobic E. coli.

However, it is probable that many bacteria have multiple means for detoxifying NO\textsuperscript{−} in their environment, and those different mechanisms, rather than being functionally redundant, will be important in different environments. The Nrf system is particularly well suited to NO\textsuperscript{−} removal, because it is located in the periplasm and so can detoxify NO\textsuperscript{−} before it enters the cell. Also, the electron transfer step from quinol to NrfA is not thought to be coupled to the generation of a proton-translocating motive force, and so the physiological turnover of NO\textsuperscript{−} will not be subject to respiratory control at the quinol-dehydrogenase step. To prevent complications in studying the contribution that Nrf can make to NO\textsuperscript{−} metabolism, we chose to use a mutant strain of E. coli deleted in the nir gene cluster that encodes a cytoplasmic siroheme nitrite reductase (32). This enzyme also reduces nitrite to ammonium and is switched on under anaerobic or micro-oxic conditions (20). It will now be intriguing both to assess whether Nir can also make a contribution to anoxic cytoplasmic NO\textsuperscript{−} metabolism and to extend the studies of the contribution nitrite reductases make to NO\textsuperscript{−} metabolism in other pathogenic bacteria that have nrf genes, which include S. typhimurium and Campylobacter jejuni (17).
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