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Cole, J A

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# Anaerobic bacterial response to nitric oxide stress: Widespread misconceptions and physiologically relevant responses

J. A. Cole

School of Biosciences and Institute of Microbiology and Infection, University of Birmingham, Birmingham, UK

**Correspondence**
J. A. Cole, School of Biosciences and Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK.
Email: j.a.cole@bham.ac.uk

## Abstract

How anaerobic bacteria protect themselves against nitric oxide-induced stress is controversial, not least because far higher levels of stress were used in the experiments on which most of the literature is based than bacteria experience in their natural environments. This results in chemical damage to enzymes that inactivates their physiological function. This review illustrates how transcription control mechanisms reveal physiological roles of the encoded gene products. Evidence that the hybrid cluster protein, Hcp, is a major high affinity NO reductase in anaerobic bacteria is reviewed: if so, its trans-nitrosation activity is a nonspecific secondary consequence of chemical inactivation. Whether the flavorubredoxin, NorV, is equally effective at such low [NO] is unknown. YtfE is proposed to be an enzyme rather than a source of iron for the repair of iron-sulfur proteins damaged by nitrosative stress. Any reaction catalyzed by YtfE needs to be revealed. The concentration of NO that accumulates in the cytoplasm of anaerobic bacteria is unknown, but indirect evidence indicates that it is in the pM to low nM range. Also unknown are the functions of the NO-inducible cytoplasmic proteins YgbA, YeaR, or YoaG. Experiments to resolve some of these questions are proposed.

**KEYWORDS**

chemical damage, enzymic activity of YtfE, hybrid cluster protein, nitrosative stress, non-physiological artefacts

## 1 | INTRODUCTION TO THE ANAEROBIC BACTERIAL RESPONSE TO NITRIC OXIDE-INDUCED STRESS

Bacteria are exposed to some level of stress in many different environments caused, directly or indirectly, by exposure to nitric oxide (NO), which binds to or reacts with many cellular components. Metalloproteins that function in electron transfer reactions, as enzymes, or as transcription factors are especially vulnerable. Exposure to NO therefore alters gene regulation and disrupts metabolism by inactivating key enzymes. Bacteria have responded by evolving extensive mechanisms to protect themselves from nitrosative damage.

The term "nitrosative stress" was first introduced by the Stamler group to describe –SH nitrosation and its consequences (Hausladen et al., 1996). However, it is now widely used to include what more correctly should be described as nitrosylative stress, which initiates with the binding of NO to a metal. Nitrosation of –SH groups requires oxidation of NO to $NO^+$ (Koppenol, 2012).

There is widespread agreement that the major protective mechanism in aerobic bacteria involves the oxidation of NO to nitrate catalyzed by the oxygenase activity of the hemoglobin-like protein,

Hmp, or related proteins (most recently reviewed by Poole, 2020). In contrast, there is significant disagreement about how anaerobic bacteria respond to NO or nitrosative stress. This mainly involves NO reduction to nitrous oxide, which minimizes trans-nitrosation of protein −SH groups and glutathione. Many natural environments are oxygen limited rather than totally anaerobic, but at [$O_2$] below 50 μM, Hmp activity declines sharply due to its far lower affinity for oxygen than cytochrome oxidases (Poole, 2020; Robinson & Brynildsen, 2016). Even in oxygen-limited environments, NO detoxification therefore requires reduction to $N_2O$ rather than oxidation to nitrate.

A major source of disagreement in the literature has been the interpretation of the physiological significance of the results of experiments in which bacteria were exposed to concentrations of NO orders of magnitude higher than those known to occur in any natural environment. Metalloproteins bind and react with NO with widely differing affinities, and exposure often causes chemical inactivation or destruction (Figure 1; range 3). Although this problem was a major focus of the review by Spiro (2007), the points made in that review are still being ignored. Chemical damage due to extreme experimental conditions has been recognized by others, for example, by Mukhopadhyay et al. (2004), who referred to the possibility of "collateral damage," and by Rowley et al. (2012).

This review will attempt to identify the dividing lines between chemical artifacts and physiologically relevant biochemical functions. Two assumptions will be made: first, that microbial metabolism has evolved to help bacteria survive under conditions that they encounter naturally; second, that gene expression is regulated to enable bacteria to adapt to environmental changes. If these assumptions are correct, knowledge of how gene expression is regulated is a good guide to the function of the gene product. Conversely, genes encoding enzymes that are known to be part of a stress response are usually regulated by a transcription factor directly involved in sensing the stress (Spiro, 2007).

## 1.1 | Major and minor sources of nitrosative stress in oxygen-limited environments

In anaerobic environments, bacteria are exposed to NO from various sources. First, NO is generated in the periplasm as an obligate intermediate in denitrification. It is also generated in the cytoplasm from nitrite that accumulates during denitrification or nitrate reduction to ammonia (Satoh et al., 1981; Smith, 1982). Bacteria associated with animal hosts are exposed to exogenous NO formed from arginine by NO synthase, an enzyme that is also present in some bacteria (Adak et al., 2002). Although NO is polar, it is soluble in water (1.94 mM in a saturated solution at 25°C). It can diffuse across the epithelial cell layer from the aerobic blood stream where it is formed into the oxygen-limited gastro-intestinal tract. Other minor or indirect sources of NO include atmospheric production of NO; and release of NO from nitrosated and nitrosylated cellular components. Whatever the source of NO exposure, the critical question is the concentration of NO to which bacteria are exposed naturally.

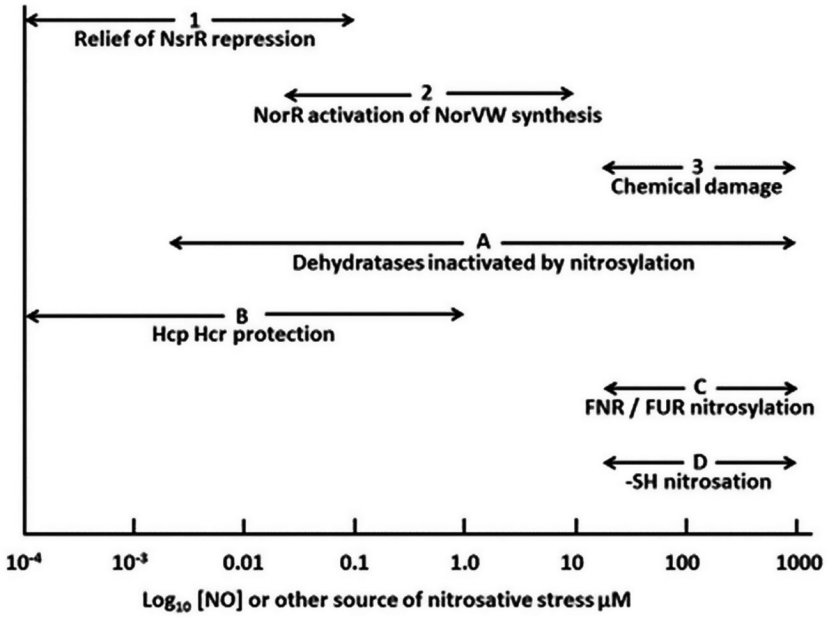


**FIGURE 1** Response of anaerobic enteric bacteria to different levels of nitrosative stress. The top three arrows indicate the likely ranges of [NO] that accumulate in the anaerobic bacterial cytoplasm (labeled 1), the range in which NorR activates the synthesis of NorVW (2) and the range at which metalloproteins are damaged chemically (3). Note that insufficient data are available to define the upper and lower limits of these ranges, so the figure is a best guess cartoon. Arrow A indicates the range of [NO] sufficient to inactivate highly sensitive [4Fe-4S] proteins such as aconitase B and fumarase B. Arrow B indicates the range over which the high affinity NO reductase Hcp is active (Wang et al., 2016). Arrows C and D indicate the much higher concentrations of NO required to inactive the transcription factors FNR, Fur, and OxyR

Much of the current information about how anaerobic non-denitrifying bacteria respond to NO is based upon experiments with *E. coli* and other *Enterobacteriaceae* in which NO is generated as a side product during the reduction of nitrite to ammonia. In these bacteria, NO production from nitrite is catalyzed mainly by the cytoplasmic nitrate reductase, NarG (Calmels et al., 1988; Ralt et al., 1988; Rowley et al., 2012; Seth et al., 2012, 2018; Smith, 1982). However, there are also other minor sources (Balasiny et al., 2018; Corker & Poole, 2003; Weiss, 2006). Under normal growth conditions, this side reaction accounts for only a very small proportion of the nitrite reduced by the dedicated nitrite reductases, NirBD and NrfAB (Smith, 1982; Wang et al., 2016). However, under extreme conditions, up to 20% of the nitrite was reduced via nitric oxide to nitrous oxide rather than to ammonia (Rowley et al., 2012). As it is unlikely that the conditions used in these experiments ever occur naturally, the authors noted that "it merits reflection whether this has any physiological importance."

## 1.2 | Concentrations of NO and its derivatives in natural environments

There are surprisingly few data in the literature of direct measurements of NO concentrations in natural environments, and even fewer estimates of the NO concentration that accumulates within the bacterial cytoplasm. These data are essential to define what is referred to below as "the physiologically relevant range" (Figure 1; range 1). In contrast, there have been many contradictory reports that the concentrations of NO, nitrosylated or nitrosated sources of NO are either high, or so low that they are physiologically irrelevant. Reports that macrophages generate micromolar concentrations of NO are repeatedly cited. However, the data on which these statements were based report only the concentration of nitrite that accumulated over periods of up to 48 hr. Nitrite accumulates gradually as the end-product of NO synthesis by iNOS followed by its chemical oxidation (see, e.g., Roy et al., 2004). Rarely were attempts made to determine the much lower steady state concentration of NO in or around mammalian cells, even under extreme conditions used to complete laboratory experiments.

The intracellular concentration of NO in mammalian cells was originally estimated to be of the order of 1 μM (reviewed by Hall & Garthwaite, 2009). Some of these estimates were based upon data from measurements with electrodes that were later shown to be subject to interference by other compounds. Subsequently, much lower estimates were obtained from various approaches that included the use of guanylyl cyclase as an endogenous NO biosensor in tissues subjected to a variety of challenges. To quote directly from Hall & Garthwaite (2009), "All these independent lines of evidence suggest the physiological NO concentration range to be 100 pM (or below) up to ~5 nM, orders of magnitude lower than was once thought."

Many pathogenic bacteria are highly resistant to antimicrobial therapy because they form biofilms (Mah & O'Toole, 2001). Formation of NO by the host is an effective defense mechanism in part because it provokes biofilm dispersal and hence increases vulnerability to antibiotic therapy. Key players in this bacterial response to NO are the hemoproteins of the H-NOX and NosP families. In many bacteria the hemoprotein H-NOX is associated with and regulates the sensor kinase of a two-component regulatory system that is activated by NO. In contrast, *Pseudomonas aeruginosa* is typical of other biofilm forming bacteria that lack an *hnoX* gene. These bacteria instead rely upon another hemoprotein, NosP, to activate the NO response (Hossain et al., 2017). Both proteins have been implicated in mechanisms that switch the formation of cyclic-di-GMP to its removal by phosphodiesterases. Description of the complex mechanism involved are beyond the scope of this review, but the relevant point is that both H-NOX and NosP respond to pM concentrations of NO that occur naturally in the human body (reviewed by Williams & Boon, 2019).

Nitric oxide generated from nitrite as a key intermediate of denitrification by α- and β-proteobacteria is reduced to nitrous oxide as rapidly as it is formed. A mutant of *Pseudomonas stutzeri* defective in the NO reductase, NorBC, was unable to grow anaerobically by denitrification because NO production catalyzed by the nitrite reductase, NirK, was toxic (Braun & Zumft, 1991). The steady state concentration of NO during nitrate denitrification by *Pseudomonas denitrificans* was 15 nM (Bakken et al., 2012). Other bacteria also maintain tight control of NO accumulation, limiting it to the range of 5 to 35 nM. As NO is reduced in the periplasm of these bacteria, the intracellular NO concentration will be much lower than this. Only a cytoplasmic NO reductase with a very high affinity for NO would be effective in preventing metalloprotein damage by these very low concentrations.

Denitrifying bacteria vary significantly in their ability to prevent NO release into their environment (Hassan et al., 2016). Nevertheless, even a relatively prolific source, *Agrobacterium tumefaciens*, transiently accumulated only 100 nM NO during the transition from aerobic growth to anaerobic denitrification. Consequently, bacteria that share their environment are exposed to low nM concentrations of NO. However, far higher concentrations have been reported in some laboratory experiments. For example, a strain of *Agrobacterium tumefaciens* was unable to adapt from oxygen-sufficient to nitrate-dependent growth because as soon as the oxygen had been depleted, the [NO] increased to 8 μM (Bakken et al., 2012). This was sufficient to inhibit growth completely until the NO had been removed by reduction to nitrous oxide, raising the question whether some bacteria accumulate much higher [NO] in their natural environments than implied above. This possibility will be discussed further at the end of this review.

Many cytoplasmic proteins in anaerobic bacteria are extremely sensitive to inactivation even by these low concentrations of NO (Gardner et al., 1997; Hyduke et al., 2007; Justino et al., 2007). Data derived from experiments with 10 or even 100 μM NO or its surrogates therefore need to be interpreted with caution because these values are at least three and possibly up to seven orders of magnitude above the physiological range and well into the range at which metalloproteins will be chemically damaged by nitrosylation and

the subsequent trans-nitrosation of protein –SH groups (Table 1; Figure 1).

## 1.3 | Genes induced by anaerobic nitrosative stress

During anaerobic growth, levels of expression of many genes change significantly in response to NO and other sources of nitrosative stress, but only a few are strongly regulated up to 10 to 100-fold (Cadby et al., 2017; Karlinsey et al., 2012; Overton et al., 2006; Rogstam et al., 2007). In *E. coli*, these genes include *hmp*, *ytfE*, *yeaR-yoaG*, *hcp-hcr*, *nrfAB*, and *norVW* (Constantinidou et al., 2006; Filenko et al., 2007; Roos & Klemm, 2006). Table 2 lists the functions of these gene products, where known, with key references. Transcription of these genes is induced by many different sources of NO that include exposure to nitrate, nitrite, NO gas, surrogates for NO such sodium nitroprusside, the NO-releasing NONOates, especially diethylamine NONOate (DEANO), or the trans-nitrosating agents S-nitrosothiol and S-nitrosoglutathione (Table 1; Crawford et al., 2016; Flatley et al., 2005; Hausladen et al., 1998; Rogstam et al., 2007; Seth et al., 2018). All of the encoded proteins except the periplasmic nitrite reductase, NrfA-NrfB, are located in the cytoplasm. The *norVW* genes encode the NO reductase, NorV, and its NADH-dependent reductase, NorW. The physiological roles of YeaR and YoaG are unknown.

**TABLE 1** Sources and surrogate sources of NO used in various studies

| Organism | Source of NO* | Reference |
|---|---|---|
| *E. coli* | 150 μM NO | Justino et al. (2005) |
| *E. coli* | 5 to 20 μM NO | Vine et al. (2011) |
| *E. coli* | 10 μM NOC–5 + 10 μM NOC–7 | Pullan et al. (2007) |
| *E. coli* | 100 μM GSNO | D'Autreaux et al. (2002) |
| *E. coli* | 200 μM GSNO | Flatley et al. (2005) |
| *E. coli* | 100 or 1,000 μM GSNO | **Mukhopadhyay et al. (2004) |
| *E. coli* | 100 μM DEANO | Seth et al. (2018) |
| *E. coli* | 1 mM S-nitrosocysteine | Hausladen et al. (1998) |
| *B. subtilis* | 500 μM SNP | **Rogstam et al. (2007) |
| *Rhodobacter sphaeroides* 2.4.1 | 1 mM GSNO or SNP | **Arai et al. (2013) |
| *B. subtilis* | 100 μM spermine NONOate | Nakano et al. (2006) |
| *S. aureus* | 1.1 mM DEANO + 10 mM NOC–12 | Richardson et al. (2006) |
| Rat muscle cells | 5 to 100 μM S-nitrosocysteine | Wolhuter et al. (2018) |

NOC-5:3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine. NOC-7:3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methly-1-propanamine.

*Abbreviations for the sources of NO used in various studies. GSNO, S-nitrosoglutathione; SNP, S-nitrosopenicillamine; DEANO, diethylamine NONOate; NONOates, NO-releasing chemicals, structure $R^1R^2N–(NO^–)–N=O$, where $R^1$ and $R^2$ are alkyl groups; **These cultures were grown with aeration.

## 1.4 | The controversial physiological role of the hybrid cluster protein

Throughout three decades of research that produced high-resolution structural and spectroscopic information, the physiological role of Hcp remained unknown. Four roles have been proposed, two of which can immediately be discarded (Hagen, 2019). A catalytically ineffective hydroxylamine reductase activity was demonstrated for the *E. coli* Hcp (Wolfe et al., 2002). Despite warnings by the authors that this was unlikely to be its physiological function, Hcp is still annotated in many genomes as a hydroxylamine reductase. For similar reasons, a proposal that it provides defense against peroxide stress can be discounted (Almeida et al., 2006; Hagen, 2019).

In macrophage experiments, Kim et al. (2003) reported that an *hcp* mutant of *Salmonella enterica* serovar Typhimurium is sensitive to NO generated from acidified nitrite. Similar results were obtained by Boutrin et al. (2012) for *Porphyromonas gingivalis*, and da Silva et al. (2015) showed that a *Desulfovibrio gigas hcpR* mutant that cannot produce Hcp is sensitive to nitrosative stress.

Wang et al. (2016) were the first to report that the *E. coli* Hcp is a high affinity NO reductase that detoxifies the low concentrations of NO that accumulate in the bacterial cytoplasm during anaerobic growth. This function is consistent with the requirement to protect cytoplasmic enzymes, especially the dehydratase family, from NO generated by NarG during nitrate reduction (Constantinidou et al., 2006; Filenko et al., 2007). Wang et al. (2016) showed that a major role of Hcr (the NADH-dependent hybrid cluster protein reductase) is to protect the NO reductase activity of Hcp from inactivation by high concentrations of its substrate, NO. These results need to be confirmed not only by other laboratories, but also for the Hcp proteins of other bacteria, especially those that lack Hcr.

Despite this evidence that the primary role of Hcp is to detoxify the very low concentrations of NO released into the bacterial cytoplasm during nitrate or nitrite reduction, a conflicting role was proposed that Hcp is an enzyme that is first nitrosylated by NO and then catalyzes nitrosation of a wide range of other proteins (Seth et al., 2018). Seventy-four mutants were constructed and the total amount of protein nitrosation during anaerobic growth in the presence of nitrate was compared with that of the parent strain. Nitrosation in the *hcp* mutant was less than 10% that of the parent, significantly less than in any of the other mutants in this study. However, decreases in protein nitrosation of more than 50% were also reported for mutants defective in *ymgA*, *ymgC*, *ybaY*, and other genes tested. Despite this and the fact that none of these other

**TABLE 2** Tanscripts most highly induced in response to nitric oxide

| Gene or operon | Function of gene products | Comments | Key references |
|---|---|---|---|
| *hcp* | High affinity NO reductase | Also high trans-nitrosylase activity | Wang et al. (2016); Hagen (2019); Seth et al. (2018) |
| *hcr* | NADH-dependent Hcp reductase, Hcr | Also protects Hcp from NO substrate inhibition | van den Berg et al. (2000)<br>Wang et al. (2016) |
| *hmp* | NO oxygenase | Also a weak associated anaerobic NO reductase | Gardner and Gardner (2002) |
| *ytfE* | Controversial: see discussion in text | Release of NO, probably indirectly, from NO damaged iron-sulfur centers | Justino et al. (2005)<br>Balasiny et al. (2018) |
| *yeaR-yoaG* | Unknown function | Strongly regulated by NsrR; weakly by NarL and NarP | Constantinidou et al. (2006)<br>Lin et al. (2007) |
| *nrfAB* | Periplasmic cytochrome *c* nitrite reductase | Electrons from the quinone pool reduce nitrite, and also NO, to ammonia | Darwin et al. (1993)<br>Wonderen et al. (2008) |
| *norVW* | NO reductase, NorV, and its reductase, NorW | Transcribed from a $\sigma^{54}$-dependent promoter: enhancer protein NorR | Hutchings et al. (2002) |

proteins were further investigated, Seth et al. (2018) concluded that Hcp is essential for protein S-nitrosation. Hcp was itself one of many proteins shown to be nitrosylated and S-nitrosated, to dimerize and be associated with many other proteins during anaerobic growth in the presence of nitrate. On this basis, Seth et al. (2018) proposed that this widespread protein S-nitrosation plays a major role in the ability of *E. coli* to resist exogenous nitrosative stress while exploiting nitrate during anaerobic growth. The conflicting claims for the role of Hcp clearly require closer analysis.

Spiro (2007) emphasized three points relevant to the current discussion. (i) Bacterial environmental sensors respond to very low concentrations of their target ligands. (ii) The products of genes that respond to the signal are specifically required for an effective stress response. (iii) Conversely, gene products that respond in a coordinated way to an environmental signal are typically part of the protective stress response that is triggered by a highly specific transcription factor. The proposal that Hcp is a high affinity NO reductase that is regulated by the NO-responsive transcription factor, NsrR, meets all of these criteria. Conversely, the proposal that the primary function of Hcp is to nitrosate protein −SH groups fails the final criterion because the majority of the nitrosated proteins play no known role in protection against nitrosative stress. Two recent papers provide possible explanations for the conflicting views summarized above.

Effective protection against NO toxicity by reduction to $N_2O$ would require a cytoplasmic NO reductase with both a very low $K_m$ for NO and a high efficiency, $kcat/K_m$. The weak NO reductase activity of the flavohemoglobin Hmp clearly fails this test (Gardner & Gardner, 2002). The only estimated catalytic efficiency of any Hcp was $2.4 \times 10^9$ $M^{-1}$ $s^{-1}$, six times higher than that estimated for NorV (Wang et al., 2016). However, the application of the Michaelis–Menten model by Wang et al. (2016) has correctly been challenged. Hagen (2019) presented spectroscopic evidence that the *D. vulgaris* Hcp is indeed an NO reductase, but the key intermediate is a dinitrosyl Fe intermediate. This provides a potential mechanism to explain how Hcp can bind and reduce 2 NO molecules to generate $N_2O$. It also provides a possible mechanistic explanation for protein −SH nitrosation catalyzed by Hcp. By analogy with other iron-sulfur proteins, Hagen (2019) proposed that the hybrid center of Hcp can exist in four redox states that he designated reduced, semi-reduced, oxidized (the form in which Hcp was isolated) and super-oxidized. Two molecules of NO can bind sequentially to either the oxidized or reduced forms of Hcp, though little, if any, mononitrosyl Hcp was detected spectroscopically even with a 2:1 ratio of Hcp to NO. In fig. 6, Hagen (2019) suggests that it is the dinitrosyl form of oxidized Hcp that can trans-nitrosylate (and hence nitrosate) other proteins: the reduced forms are required for NO reductase activity. This would explain the role of Hcr reported by Wang et al. (2016) in protecting the NADH-dependent NO reductase activity by reducing the oxidized to the reduced forms. This proposal is thermodynamically far more plausible than the role proposed by Seth et al. (2018) that Hcr enables $NAD^+$ to serve as an electron acceptor for the oxidation of NO to $NO^+$. However, unless Hcp is unique, many other iron-sulfur proteins would also be nitrosylated by the same mechanism: unlike its primary role as an NO reductase, the targets for nitrosation by Hcp would include proteins unrelated to the nitrosation stress response, as reported by Seth et al. (2018). Furthermore, unlike NO reduction, −SH nitrosation would be nonspecific, explaining why many proteins in addition to Hcp also catalyze nitrosation (Figure 1a of Seth et al., 2018.

The paper by Hagen (2019) provided the first insight into a possible mechanism for the two contrasting activities of Hcp. Bulot et al. (2019) showed that during anaerobic growth in the presence of nitrate, both the nitrate reductase, NarGHI, and its cognate formate dehydrogenase, FdnGHI, cluster at the poles of the bacteria, consistent with the formation of a multi-protein complex. Cross-linking experiments revealed the presence of possible interacting proteins that included Hcp. However, the same components

clustered at cell poles even in an *hcp* mutant, so contrary to the proposal of Seth et al. (2018), complex formation was neither dependent upon nor driven by Hcp. Significantly, other proteins induced in response to NO stress such as NorVW also co-located with NarGHI and FdnGHI only in the absence of Hcp. This is strong evidence that Hcp provides the first line of defense against NO stress. When conditions become oxidizing and Hcr can no longer protect it, Hcp becomes oxidized and inactivated by nitrosation and synthesis of NorVW is induced by the higher concentration of NO in the bacterial cytoplasm.

Four experiments would resolve any remaining doubts about the primary physiological role of Hcp. (i) First, it is predicted that there would be minimal protein nitrosation under the physiologically relevant conditions used in the experiments described by Wang et al. (2016), but far more in an *hcp* mutant than in the *hcp*$^+$ parent. Conversely, the NO reductase activity of Hcp is predicted to be inactivated under the growth conditions described by Seth et al. (2018). (ii) The relative kinetics of NO reduction and protein nitrosation by the dinitrosyl intermediate also need to be determined. (iii) The 74 mutants assayed for nitrosation activity were selected from genes upregulated during nitrate respiration more in the parent strain than in an *oxyR* mutant. As the *fdn* genes were not in this list, it would be interesting to determine the effects of an *fdn* mutation on nitrosation activity and whether FdnGHI also catalyze trans-S-nitrosation. (iv) Finally, it is predicted that in chemostat competition experiments, if Hcp is physiologically the more important NO reductase at very low concentrations of NO, an *hcp*$^+$*norVW* mutant would outcompete an *hcp norVW*$^+$ mutant during anaerobic, nitrate limited growth. In contrast, when nitrate is in excess, the NO reductase activity of Hcp would be inactivated by nitrosation, so the *hcp norVW*$^+$ mutant would have the competitive advantage.

## 1.5 | Transcription factors that regulate gene expression in response to NO

All of the well-characterized transcription factors that regulate the bacterial response to NO are metalloproteins. There have been many reports of activators and repressors that fall outside this generalization, but most of the original claims have subsequently been challenged. Until independent experimental confirmation has been published, the initial claims remain controversial. Transcription factors that respond directly and with high sensitivity to NO fall into two classes: hemoproteins; and nonheme iron or iron-sulfur proteins (Rodionov et al., 2005). Typical of the former group are the HcpR proteins of sulfate reducing bacteria and *Porphyromonas gingivalis* (Belvin et al., 2019; Cadby et al., 2016; da Silva et al., 2015). The second group includes NsrR proteins found in a wide range of β, γ, and δ proteobacteria, the enhancer binding protein, NorR, and other HcpR proteins (Crack et al., 2011; D'Autréaux et al., 2005; Isabella et al., 2009; Pohlmann et al., 2000; Yuki et al., 2008). In some bacteria there are multiple HcpR genes. For example, in *D. desulfuricans* 27,774, heme is the NO-sensing ligand in HcpR1. A [4Fe-4S] center in HcpR2 detects NO and induces *hcp* expression (Cadby et al., 2016; da Silva et al., 2015).

The SrrAB two-component system of *Staphylococcus aureus* undoubtedly plays an important role in the defense against nitrosative stress (Richardson et al., 2006). However, the SrrAB regulon extends well beyond genes specifically involved in the nitrosative stress response, so it is unlikely that the environmental sensor, SrrA, detects NO directly (Kinkel et al., 2013).

In *Campylobacter jejuni*, synthesis of the globins Cgb (a single-domain globin) and Ctb (a truncated globin) is induced in response to NO via the positively acting transcription factor, NssR. These are also indirect effects rather than a transcriptional response initiated by the binding of NO to NssR. Similar arguments apply to the thiol-based RNA polymerase regulatory protein, DksA, in *Salmonella* (Crawford et al., 2016).

## 1.6 | How transcription control mechanisms reveal the roles of gene products

Two contrasting transcription factors, NorR and NsrR, regulate the response of *E. coli* to NO. The NO reductase NorV has a high affinity for NO (estimated $K_D$ for NO < 1 μM; Gomes et al., 2002), The *norVW* operon is expressed from a sigma$^{54}$-dependent promoter and is totally dependent upon activation by the binding of NO to the enhancer binding protein, NorR (Büsch et al., 2002; D'Autréaux et al., 2005; Gardner et al., 2002; Hutchings et al., 2002; Pohlmann et al., 2000). D'Autréaux et al. (2005) estimated the $K_D$ for NO dissociation from NorR to be 50 ± 10 nM. The NorVW operon is therefore expressed under conditions of high NO stress (Bulot et al., 2019; Hall & Garthwaite, 2009; Wang et al., 2016). Significantly, *norV* and *norR* mutants of *E. coli* were not compromised for survival in macrophages (Pullan et al., 2007). These observations strongly support the proposal that Hcp rather than NorV provides the main protection mechanism for cytoplasmic proteins under normal levels of nitrosative stress. Pullan et al. (2007) also demonstrated that nitrosative stress is due to metalloprotein NO nitrosylation rather than to −SH nitrosation reactions.

In contrast to *norVW*, expression of other genes induced by NO are repressed by NsrR. They are transcribed from promoters dependent upon the housekeeping sigma factor, $\sigma^{70}$. This reflects the contrasting roles of the two major *E. coli* transcription factors NsrR and NorR that respond to nitrosative stress. Genes regulated by the $\sigma^{70}$-dependent NsrR provide a first line of defense against the very low concentrations of NO that occur in the bacterial cytoplasm (Figure 1, range 1). The housekeeping function of NsrR-regulated gene products is to protect vulnerable cytoplasmic proteins such as the dehydratase family against the very low concentrations of NO encountered during normal growth (Duan et al., 2009; Hyduke et al., 2007; Ren et al., 2008; Varghese et al., 2003). When the intracellular [NO] increases toward the concentration that would overwhelm NsrR-regulated defenses, NorR is activated and the NorVW

system provides an effective stress response (Figure 1; range 2: see also Chismon et al., 2010; Karlinsey et al., 2012). Insufficient data are available to define the lower limits of cytoplasmic [NO] or external nitrosative stress required to inactivate NsrR or activate NorR by nitrosylation.

The *hcp-hcr* and *nrfAB* operons are expressed only during anaerobic growth and require a functional FNR protein, but expression of *hmp* is repressed by FNR. This is consistent with the oxygen-dependent role of Hmp as an NO oxygenase during aerobic growth, but not during anaerobic growth (Gardner & Gardner, 2002). The *hcp* operon is strongly induced during nitrate and nitrite reduction to ammonia (Constantinidou et al., 2006; Vine et al., 2011). This established two important points. First, NsrR is far more sensitive to NO than FNR. Second, FNR is almost fully active even during anaerobic growth in the presence of concentrations of nitrate or nitrite above those encountered in natural environments (Figure 1, ranges A and C). Nevertheless, expression from NsrR-regulated promoters is fully derepressed in an *nsrR* mutant, but only partially derepressed during anaerobic growth in the presence of nitrate or nitrite (Vine et al., 2011). NsrR from *Streptomyces coelicolor* binds to its DNA target sites with very high affinity (Crack et al., 2016). Assuming the same is true for *E. coli* NsrR, this is further evidence that the concentration of NO in the *E. coli* cytoplasm is extremely low during nitrate or nitrite reduction, well below the $K_D$ of NsrR for NO and consistent with a cytoplasmic NO concentration in the low nanomolar or picomolar range, as estimated by Wang et al. (2016) and others.

Excess nitrate induces expression of the cytoplasmic nitrate reductase operon *narGHJI* and the nitrate-nitrite transporter gene, *narK*. As nitrite reduction to NO by NarG is the major source of NO in the cytoplasm, it makes physiological sense for the nitrite reductases, NrfAB and NirBD, as well as Hcp, to be regulated by FNR coordinately with nitrate uptake and reduction. Despite its high Km for NO, the high activity of the periplasmic nitrite reductase provides a first line of defense against NO originating outside the bacteria (Wonderen et al., 2008).

NO reduction by α- and β-proteobacteria is largely regulated by proteins of the Fnr/Crp family (Rodionov et al., 2005). In contrast, the NO reductase genes of the truncated denitrification pathway of pathogenic Neisseria are regulated by NsrR (Heurlier et al., 2008; Overton et al., 2006). This again makes physiological sense because one source of nitrosative stress in the human body is NO generated in aerobic tissues from arginine by NO synthases. NO is also a product of nitrite reduction in oxygen-limited tissues. As nitrite reduction provides an electron acceptor for energy generation when oxygen is scarce, expression of the nitrite reductase gene, *nirK*, is regulated by both FNR and NsrR. These are therefore examples of how gene regulation has evolved to enable bacteria to exploit their anaerobic environment. They illustrate how physiological roles can be revealed by knowledge of transcription control mechanisms. Conversely, transcription factors that regulate genes known to be involved in NO metabolism are likely to be *bona fide* NO sensors (Spiro, 2007).

## 1.7 | Errors in the assignment of physiological roles due to protein damage by unnatural levels of stress

Many misunderstandings of how nitrosative stress is regulated have arisen because the bacterial transcription factors that regulate responses to a lack or excess of redox-active metals, oxygen and reactive oxygen species are iron or iron-sulfur proteins. At high concentrations of NO or related species, these other transcription factors are inactivated chemically and are therefore unable to function. This results in derepression of some genes and failure to express others, but only under extreme conditions rarely encountered by the bacteria in which they have evolved (Spiro, 2007). A remaining source of controversy is whether there is a dividing line between irrelevant chemical damage that requires extreme conditions never encountered naturally, and a physiological response that confers a survival advantage under conditions of stress (Figure 1, ranges 2 and 3). The FNR protein provides an excellent example.

FNR is continuously synthesized and the housekeeping Isc iron-sulfur assembly apparatus inserts the oxygen-sensing [4Fe-4S] iron-sulfur center into the apoprotein. Binding of FNR to its DNA target requires an intact [4Fe-4S] center. The binding of oxygen to this center results in destruction of the [4Fe-4S] center, loss of DNA binding and hence loss of FNR-dependent transcription activation and repression. The first step in the inactivation of FNR by oxygen is the loss of iron from the [4Fe-4S] cluster (Green et al., 1996; Sutton et al., 2004). Although the same is true of inactivation by NO of dehydratases such as the dihydroxy-acid dehydratase, IlvD, aconitase and fumarase, these iron-sulfur proteins are much more sensitive than FNR to nitrosylative inactivation (Figure 1, ranges A and C; Duan et al., 2009; Hyduke et al., 2007; Ren et al., 2008; Varghese et al., 2003; Wimpenny & Cole, 1967). The roposal that FNR is also an environmental sensor of NO was based on experiments in which the concentration of NO used was sufficient to destroy its iron-sulfur center, thus, inactivating its physiological function of repressing Hmp synthesis during anaerobic growth (Figure 1, range C; Cruz-Ramos et al., 2002). The *hmp* gene is repressed both by FNR and also by NsrR. Almost all of the response to NO in this original report were subsequently shown to be due to relief of repression by NsrR rather than to relief of FNR repression (Bodenmiller & Spiro., 2006; Pullan et al., 2007).

The primary role of the SoxRS two-component regulatory system is to regulate the response to superoxide stress, but SoxR also binds NO (Nunoshiba et al., 1993). However, SoxRS does not regulate genes required for NO defense mechanisms and SoxR is not an NO sensor.

In many bacteria iron uptake and metabolism are regulated by the iron protein, Fur. As Fur, like FNR, can be inactivated by NO nitrosylation, it has incorrectly been assigned a role in the response to nitrosative stress (D'Autreaux et al., 2002). This conclusion is again based upon data from laboratory experiments under conditions unlikely to be encountered by bacteria in their natural environments (Figure 1; range C).

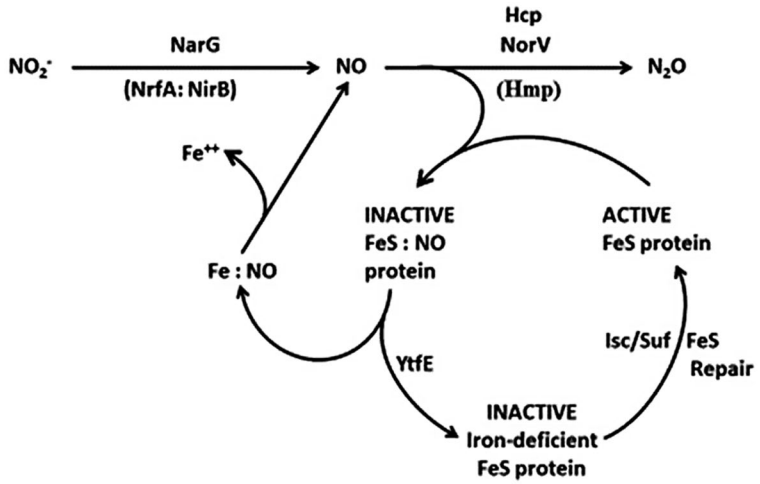


**FIGURE 2** An alternative possible model for the repair of nitrosylated iron-sulfur proteins by YtfE. The model suggests that the nitrosylated iron is released with NO attached. The released product might be Fe:NO or Fe:$(NO)_2$, which can then dissociate to free NO and $Fe^{++}$. Whether an enzyme might be required to dissociate the iron atom from Fe:NO is unknown. The released NO is available for reduction by the Hcp-Hcr NO reductase. The inactive iron-deficient Fe-S protein can then be repaired in vivo by the Isc or Suf iron-sulfur center assembly proteins

Seth et al. (2012) reported that S-nitrosation of cysteine 199 during anaerobic nitrosative stress converts *E. coli* OxyR from a transcription factor that regulates oxidative stress to a regulator of nitrosative stress. Rates of transcription of 145 genes were higher in the presence of nitrate than with fumarate: 129 of these transcripts were less abundant in an *oxyR* mutant than in the parent strain. No bioinformatics analysis of the promoter regions of these transcripts was reported, so no consensus sequence for a nitrosated OxyR-binding site was proposed. The possibility was recognized that the effects of OxyR might be either direct at some promoters, but indirect at others. Transcription of the *hcp-hcr* operon was induced only 5-fold during growth of an *oxyR* mutant in the presence of nitrate relative to growth with fumarate compared with a 40-fold increase in the parent strain. With this exception, there was little correlation between transcripts dependent upon OxyR for induction during anaerobic growth and products known to be involved in protection against NO or nitrosative stress (Figure 2 of Seth et al., 2012). Although OxyR was shown to bind and recruit RNA polymerase to the *hcp* promoter, the OxyR-binding site was not identified. Clearly further experiments are required to determine whether S-nitrosated OxyR is a physiologically significant regulator of an *E. coli* nitrosative stress response (see also Hausladen et al., 2012 and earlier references cited therein).

### 1.8 | The physiological role of YtfE in the repair of nitrosative damage

Dinitrosylation of some iron-sulfur proteins, for example, the dehydratase family, results in chemical damage and the slow release of iron atoms (D'Autreaux et al., 2002; Duan et al., 2009; Hyduke et al., 2007). This free iron then becomes available to bind to other iron-deficient proteins. The di-iron protein YtfE (also known as RIC, for the repair of iron-sulfur centers) has been shown in vitro to be able to donate iron to apo-ferredoxin and to the iron-sulfur assembly protein, IscU. It also exchanges protein-bound iron with free iron (Justino et al., 2007; Nobre et al., 2014). These processes were extremely slow, requiring incubation periods of 15 to 75 min and higher than stoichiometric concentrations of YtfE than the protein to be reconstituted. This raised the possibility that there is an alternative, physiologically more relevant function of YtfE. A crystal structure of YtfE revealed the presence of two solvent-accessible channels, both of which converge to the di-iron center and might therefore be critical for capturing substrates (Lo et al., 2016). The authors demonstrated the ability of their purified protein to reduce NO to $N_2O$, but the reaction was too slow to account for the rates of NO reduction detected with bacterial suspensions.

We recently reported that YtfE directly or indirectly releases NO from nitrosylated iron-sulfur centers (Balasiny et al., 2018). Two surprising results were reported. First, a $YtfE^+$ $Hcp^-$ strain was far more sensitive to growth inhibition by NO than the isogenic $Hcp^-YtfE^-$ strain. Second, neither aconitase nor fumarase were more active in strains in which YtfE was functional than inactive. This failure of YtfE to reactivate aconitase and fumarase in the mutant might be explained in either of two ways. One possibility, previously published by Balasiny et al. (2018), is that NO is released directly from nitrosylated iron-sulfur proteins by YtfE. In the absence of Hcp, the NO released immediately re-nitrosylates iron-sulfur centers of enzymes like aconitase and fumarase, effectively establishing a futile repair cycle. The alternative explanation might be that NO is released attached to the nitrosylated iron atom, leaving an inactive iron-deficient enzyme. If so, the iron deficiency could then be repaired by the Suf or Isc pathway, NO being released by dissociation from the Fe-NO or Fe-$(NO)_2$ produced (Figure 2). Although *hcp* and *ytfE* transcription are both repressed by NsrR, they are not regulated coordinately by Fnr. Does this indicate that YtfE has roles wider than repairing nitrosylative damage? Further experiments are clearly required to identify the primary product of YtfE action and to resolve which proposed function of YtfE is correct. Exposure of YtfE (RIC) to an exceptionally high concentration of NO would probably result in the loss of any physiological relevant function. It will therefore be essential that future experiments avoid exposure to high concentrations of NO that might inactivate any physiologically relevant enzyme activity.

## 2 | CONCLUSIONS AND SOME OF THE MANY UNANSWERED QUESTIONS

Quantitative data on the concentration of NO in the bacterial cytoplasm are still unavailable. However, levels of transcription of NsrR-repressed genes are far higher in an *nsrR* mutant than in the parent

strain during anaerobic growth in the presence of nitrite or NO, so in the latter case, it is below the $K_D$ of NsrR for NO. Unexplained is why some NsrR-regulated genes are more effectively induced during anaerobic growth in the presence of nitrite than with a high concentration of NO. For example, *yeaR-yoaG* transcripts are far more abundant after anaerobic growth in the presence of nitrite than after exposure to high [NO] (for details, compare data reported in Pullan et al., 2007 and Vine et al., 2011 with Constantinidou et al., 2006). As NO is freely diffusible, is this simply the consequence of extreme nitrosylative damage to proteins required for the adaptive response? If not, the highly unlikely alternative is that there is a barrier that prevents external NO from equilibrating with the cytoplasmic concentration. Any such barrier remains undefined.

It was argued above that nitrosation of −SH groups is only a minor problem for anaerobic bacteria. However, Hungate tubes are no more a natural environment than growth in the presence of DEANO. Most anaerobic bacteria need to be able to survive and even exploit exposure to low concentrations of oxygen that might be sufficient to serve as electron acceptor for the metal-dependent oxidation of NO to the required nitrosating agent, $NO^+$. It would be interesting to know the levels of protein S-nitrosation in bacteria in natural environments, for example, in wastewater treatment plants where nitrate can be abundant but electron donors are scarce. Roos and Klemm (2006) reported that nitrate is sufficiently abundant in the microaerobic environment of the human urinary tract to support growth of an *E. coli* infection by nitrate respiration. Synthesis of both the nitrate reductase, NarG, and NO detoxification enzymes were induced. This would provide an excellent source of bacteria to assess the level of protein −SH nitrosation in bacteria in a natural oxygen-limited environment.

Other unresolved questions include the physiological roles of YgbA, YibI-YibH, and YeaR-YoaG, proteins in enteric bacteria that are synthesized in response to nitrosative stress. Do any of them provide links between NO-sensing, biofilm dispersal, repair of DNA damage, or quorum sensing and loss of motility? Can we correlate their induced synthesis with specific sources of stress, for example, NO generated internally during nitrate reduction or exposure to external NO?

It is highly likely that YtfE (RIC) is an enzyme, but the reaction it catalyzes remains to be established. Both the sensitivity of this activity to high concentrations of NO and the form in which the NO is released need to be identified. The proposal that it has a physiological role in repairing nitrosylated iron or iron-sulfur centers seems increasingly unlikely to be correct (Lo et al., 2016). If, however, it is correct, how substrate specific is YtfE repair? Both of the NsrR-repressed operons, *ytfE* and *yeaR-yoaG* are more highly expressed in an *fnr* mutant than in the $fnr^+$ parent (Constantinidou et al., 2006). They are also regulated by NarL. No FNR binding site is present in their promoter regions, so the regulatory mechanisms remain to be revealed.

Independent confirmation is urgently required that Hcp is a high affinity NO reductase in bacteria other than *E. coli*. Are proteins other than Hcr, for example, a cupin, physiological electron donors to Hcp? What is the electron donor to Hcp in bacteria that lack Hcr? Is Hcp inactivated by oxygen and, if so, does NorV dominate NO reduction in microaerobic environments? At sub-μM [NO] that occur naturally, what are the relative contributions of Hcp and NorV to NO reduction?

This review has demonstrated how misconceptions have arisen because they were based upon experiments under physiologically irrelevant conditions. High concentrations of NO or surrogate sources of NO are substrates for chemical reactions that rarely occur in natural biological systems. This alone is sufficient to explain misconceptions that FNR, OxyR, and Fur are NO sensors rather than transcription factors dedicated to the regulation of gene expression in response to oxygen, peroxide, or iron availability. It also led to the misconceptions that Hcp provides defense to reactive oxygen species, that it is a hydroxylamine reductase, or essential for trans-nitrosation; or that YtfE (RIC) is primarily an Fe donor that repairs damaged iron-sulfur centers. Hopefully, it has set some basic principles that must be followed to resolve the many unanswered questions.

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## ORCID

*J. A. Cole* https://orcid.org/0000-0003-0015-9962

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