Legless pathogens: how bacterial physiology provides the key to understanding pathogenicity

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This review argues that knowledge of microbial physiology and metabolism is a prerequisite to understanding mechanisms of pathogenicity. The ability of Neisseria gonorrhoeae to cope with stresses such as those found during infection requires a sialyltransferase to sialylate its lipopolysaccharide using host-derived CMP-NANA in the human bloodstream, the ability to oxidize lactate that is abundant in the human body, outer-membrane lipoproteins that provide the first line of protection against oxidative and nitrosative stress, regulation of NO reduction independently from the nitrite reductase that forms NO, an extra haem group on the C-terminal extension of a cytochrome oxidase subunit, and a respiratory capacity far in excess of metabolic requirements. These properties are all normal components of neisserial physiology; they would all fail rigid definitions of a pathogenicity determinant. In anaerobic cultures of enteric bacteria, duplicate pathways for nitrate reduction to ammonia provide a selective advantage when nitrate is either abundant or scarce. Selection of these alternative pathways is in part regulated by two parallel two-component regulatory systems. NarX–NarL primarily ensures that nitrate is reduced in preference to thermodynamically less favourable terminal electron acceptors, but NarQ–NarP facilitates reduction of limited quantities of nitrate or other, less favourable, terminal electron acceptors in preference to fermentative growth. How enteric bacteria repair damage caused by nitrosative and oxidative damage inflicted by host defences is less well understood. In both N. gonorrhoeae and Escherichia coli, parallel pathways that duplicate particular biochemical functions are far from redundant, but fulfil specific physiological roles.

Origins of studies of bacterial physiology and pathogenicity

My research career began in October 1963 as a student in the MRC Microbial Physiology unit of Sir Hans Krebs at the University of Oxford. One question we were often asked was: what is microbial physiology? My answer then and now is that microbial physiology is the study of structure–function relationships in micro-organisms, especially how microbes respond to changes in their environment.

My PhD supervisor was David Hughes who was a remarkable character for many reasons. Before World War II he was expelled from the British Communist Party because he was too radical. He was a prolific source of ideas, and he moved rapidly from one project to another. While on sabbatical at Dartmouth College, New Hampshire, USA, in the laboratory of Clark Gray, he and a student Julian Wimpenny made the surprising discovery that Escherichia coli can synthesize cytochrome c, but only during anaerobic growth (Gray et al., 1966). A few years earlier, another father figure of British microbiology, John Postgate, had discovered cytochrome c in strictly anaerobic sulphate reducing bacteria (Butlin & Postgate, 1953; Postgate, 1956). This was a seminal discovery because cytochromes had been linked to respiration, and the dogma was that only aerobes can respire – so why do anaerobes need cytochromes? The E. coli discovery was also remarkable because it was already known that the aerobic respiratory chain of E. coli lacks both the soluble cytochrome c and the bc1 complex of the mammalian respiratory chain. My task was to determine whether this cytochrome was involved in hydrogen production during fermentation, but by the end of my PhD we knew it was involved in nitrite reduction.

Molecular mimicry as a basis of gonococcal pathogenicity

My interest in microbial pathogenicity developed much later, in January 1987. At that time, one of the key questions was why some bacteria are pathogens and others are completely harmless. In short, what determines pathogenicity? Some people then stated that pathogenicity determinants are genetic traits that enable a pathogen to damage its host; others defined them as genetic traits that confer a selective advantage on the pathogen relative to commensal bacteria.

Harry Smith, the guru of microbial pathogenicity, had been struggling since 1970 to understand how Neisseria
The gonococcus is an obligate human pathogen that is not a particularly adaptable organism. In their natural habitat, gonococci are surrounded and out-numbered by lactobacilli that convert sugars like glucose to lactic acid. Lactobacilli also reduce oxygen to hydrogen peroxide, so gonococci are surrounded and out-numbered by lactobacilli. As they live where glucose and oxygen are scarce, but lactic acid is abundant, gonococci use lactate in preference to glucose as the preferred source of carbon and energy for growth (Morse & Bartenstein, 1974). In cases of disseminated gonorrhoea, they cross the epithelial cell layer into the bloodstream where they are exposed to high concentrations of oxygen as well as the oxidative and nitrosative responses of the human host. The gonococcus can also exploit traces of nitrite found in the human body (Knapp & Clark, 1984). Outstanding questions 10 years ago were how gonococci generate energy for growth when oxygen is very scarce, and how they cope with reactive oxygen and reactive nitrogen species generated by not only their own metabolism but also their human host and other bacteria that share their environment.

Since Steve Morse’s work in the 1970s (Morse, 1979), there had been very few papers describing mechanisms of energy generation in the gonococcus, and they all reached completely contradictory conclusions. The first genome sequence revealed that all of them were completely wrong. From the genome sequence we now know that electrons pass from NADH and other physiological substrates through the cytochrome bc1 complex to the terminal oxidase, where oxygen is reduced to water. The gonococcus has only one terminal oxidase, which is a cytochrome cbb3-type oxidase. This complex has been characterized from other sources (Preisig et al., 1993; Thöny-Meyer et al., 1994; Rey & Maier, 1997; Pitcher & Watmough, 2004); it has a very high affinity for oxygen that is effective in scavenging low concentrations of oxygen.

Gonococci synthesize large quantities of eight different c-type cytochromes. Two of them are found in the terminal oxidase, and a third is the cytochrome c1 component of the bc1 complex. In most bacteria, a soluble c-type cytochrome shuttles electrons from the bc1 complex to the terminal oxidase, but in the gonococcus two cytochromes fulfil this role: cytochromes c4 and c5. These cytochromes form two parallel pathways (Li et al., 2010).

Gonococci can reduce oxygen extremely rapidly, at the same rate as E. coli. Mutants defective in either cytochrome c4 or c5 also reduce oxygen almost as rapidly and grow at almost the same rate as the parent. A possible reason why gonococci have such very high respiration rates and maintain duplicate pathways to oxygen becomes apparent only during exposure to high levels of aeration. Under these conditions, the parent strain grows much more rapidly than under oxygen-limiting conditions: it can adapt. However, when the electron transfer capacity is decreased by deleting one or other of the electron transfer pathways to oxygen, the bacteria now kill themselves because they cannot cope with the ROS that are generated as side products of respiration (Fig. 1). In short, the duplicated pathways are not redundant; they enable the gonococcus to avoid the generation of toxic ROS and they also provide protection against the oxidative burst of the host in cases of disseminated gonorrhoea.

Gonococcal survival during oxygen starvation

When oxygen is scarce, gonococci reduce traces of nitrite to nitric oxide. This one-electron reduction is catalysed by a copper-containing nitrite reductase, a member of the NirK family of proteins that, in the gonococcal literature, is widely referred to as AniA. Nitrite reduction occurred only when the bacteria were essentially anaerobic and there are antibodies to the nitrite reductase in the serum of gonorrhoea patients (Clark et al., 1987, 1988). This was the first clear evidence that gonococci must be able to
survive periods of oxygen starvation in vivo. As nitric oxide is a reactive nitrogen species it must be detoxified. This is the role of the nitric oxide reductase NorB, which reduces nitric oxide to nitrous oxide.

In enteric bacteria that reduce nitrate via nitrite to ammonia, nitrate and nitrite reduction are co-ordinately regulated by FNR, which activates transcription only in the absence of oxygen. Neisseria are the only denitrifying bacteria commonly found in the human body, and they also use FNR to ensure that the nitrite reductase gene is expressed only in the absence of oxygen. However, it would be fatal if they could synthesize nitric oxide reductase only during anaerobic growth. If they did this, how would they protect themselves against nitric oxide generated as part of the nitrosative burst of the aerobic host?

Members of the genus Neisseria have solved the problem by regulating nitrite reduction independently of nitric oxide reduction. Synthesis of the nitric oxide reductase NorB is regulated by the repressor protein NsrR (Lissenden et al., 2000; Householder et al., 2000; Overton et al., 2006; Rock et al., 2007; Whitehead et al., 2007). In the presence of NO, NsrR repression is relieved and NorB is synthesized (Fig. 2). Most soil denitrifying bacteria also induce norBC expression in response to nitric oxide, mediated by the NNR protein, which is another member of the FNR family of proteins (Zumft, 1997; Mesa et al., 2003).

### Outer membrane redox proteins as the first line of defence against chemical attack by the host

The aerobic respiration system and the nitric oxide reductase are situated on the inner membrane, allowing electrons to reach these components from NADH. However, major chemicals generated by host defences are hydrogen peroxide and nitric oxide, which therefore attack bacteria from the outside. Gonococci defend themselves against attack from outside the cell by synthesizing a range of outer membrane redox proteins. These include an inducible cytochrome c peroxidase CCP and a constitutive c-type cytochrome, cytochrome c’ or CycP, which binds nitric oxide (Turner et al., 2003, 2005). CycP is believed to act as a buffer system that limits damage by NO while the norB expression is induced (Anjum et al., 2002). Both the nitrite reductase

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**Fig. 1.** Effect of different levels of aeration on growth of cccA, cycA and cycB mutants compared with the parental strain, *N. gonorrhoeae* strain F62. Strain F62 and mutants defective in cytochrome c4 (strain JCGC800), cytochrome c5 (JCGC850) or cytochrome c2 (JCGC851) were grown in either 60 ml (a; limiting aeration) or 10 ml (b; oxygen sufficient) of GC broth in 100 ml conical flasks. Cultures were shaken at 100 r.p.m. and incubated at 37 °C. The amount of inoculum was adjusted so that each culture started with the same concentration of cells. OD650 1.0 corresponds to a biomass of 0.4 mg ml⁻¹. The figure is reproduced with permission from Li et al. (2010) (Copyright American Society for Microbiology).

**Fig. 2.** Regulation of the truncated denitrification pathway in the genus Neisseria. In progression from the top to the bottom of the figure are the two environmental signals, oxygen and nitric oxide; genes encoding the transcription factors, NarQ–NarP, FNR and NsrR; target genes encoding the nitrite reductase AniA and the NO reductase NorB; and at the bottom the two-step pathway for the reduction of nitrite via NO to nitrous oxide. Green arrows between the transcription factors and the genes they regulate indicate transcription activation; red arrows indicate transcription repression. Oxygen inactivates FNR, preventing induction of the nitrite reductase; in contrast, NO inactivates NsrR to derepress expression of norB. DnrN is the gonococcal orthologue of *E. coli* YfeE.
AniA and the lipid-associated azurin Laz, which can protect the gonococcus against ROS, are also localized on the outer membrane, so the outer membranes of these pathogenic bacteria contain an array of lipoproteins that enable them to survive oxidative and nitrosative stress. However, exactly the same is true of non-pathogenic commensal bacteria such as Neisseria lactamica or Neisseria cinerea. As all of these lipoproteins are important for survival in the human body, a critical question is how electrons cross the periplasm to reach these outer membrane redox proteins. It was during our investigations into oxygen reduction by the gonococcus that a fascinating property of the terminal oxidase came to our attention (Tovell, 2008; Hopper et al., 2009; Aspholm et al., 2010).

The cbb3 oxidase is found in many types of bacteria. It contains four subunits, CcoNOPQ. The O and P subunits are c-type cytochromes, but the gonococcal CcoP subunit is larger than CcoP in other organisms due to a C-terminal extension. It has an additional haem-binding motif which allows CcoP to bind three rather than two haem groups. To determine whether this third haem group of CcoP could be acting as an electron donor to AniA in the gonococcus, we used site-directed mutagenesis to mutagenize the third haem-binding motif of CcoP. The mutations had no effect on aerobic growth or oxygen reduction rates, and therefore this extra haem group was not involved in the transfer of electrons to oxygen. However, when mutants were grown anaerobically with nitrite as a terminal electron acceptor, they grew more slowly than the parent strain and reduction of the nitrite added to the culture was also delayed (Fig. 3). Rates of nitrite reduction by washed bacteria using lactate as the electron donor were about 50 % of that of the parent, confirming that the third haem group of CcoP is indeed involved in the transfer of electrons to AniA in the outer membrane (Hopper et al., 2009; Aspholm et al., 2010). However, clearly there must be at least one additional electron transfer pathway.

The third haem-binding domain of CcoP is highly similar to the second haem domain of cytochrome c5. We speculated that the pathway of electron transfer to AniA could perhaps pass through cytochrome c5 as well as the third haem group of CcoP. We therefore combined the CcoP third haem group mutation with a truncated version of cytochrome c5 from which the second domain containing the region of homology to the third haem group of CcoP had been deleted. The rate of nitrite reduction by the double CcoP c5 mutant was only 10 % of that of the parent strain, and over fourfold lower than the CcoP single mutant. However, the double mutant still reduced nitrite at a rate significantly higher than an AniA deletion mutant (A. Hopper & J. A. Cole, unpublished results). To summarize, there are at least three pathways for transferring electrons to the nitrite reductase: two of them are known to involve the novel third haem group of the CcoP protein and the second domain of cytochrome c5. The third pathway remains to be identified. So gonococci have evolved

Fig. 3. Growth, nitrite reduction during growth, rates of nitrite reduction by washed bacteria and accumulation of AniA protein during oxygen-limited growth of the parent strain and mutants with substitutions in the third haem group of CcoP. (a) Growth of strain F62 kan^R (▲) and mutants with the substitutions CcoP C368A (□) or C371A H372A (●). Arrows indicate the addition of 1 and 5 mM NaNO₂ 1 and 2 h after commencement of growth, respectively. (b) Concentration of nitrite remaining in the growth medium for the cultures shown in (a). (c) Rates of nitrite reduction by harvested bacteria incubated in the presence of lactate and nitrite. Data shown are the mean ± SEM of at least two assays of samples from at least two independent growth experiments, and are given in nmol NO₂ reduced min⁻¹ (mg dry cell mass)⁻¹. The figure is based upon Fig. 4 of Li et al. (2010) and is reproduced with permission (Copyright American Society for Microbiology).
Multiple electron transfer pathways to both cytochrome oxidase and to nitrite reductase.

**Repair of nitrosative damage**

A key protein involved in the repair of nitrosative damage in *E. coli* is variously called YtfE (product of the gene of unknown function, *ytfE*) or RIC, for the repair of iron centres of metalloproteins damaged by oxidative or nitrosative stress (Justino et al., 2005, 2006, 2007; Todorovic et al., 2008; Vine et al., 2010). There is an orthologue of YtfE in the gonococcus called DnrN. Its synthesis, like that of the nitric oxide reductase NorB, is induced in the presence of NO by inactivation of the transcription factor, NsrR (Overton et al., 2008). NsrR is itself an iron–sulfur protein (Tucker et al., 2008). As DnrN is a di-iron protein, if it repairs proteins damaged by nitrosation, it should also repair the damage to the iron–sulfur centre of NsrR to restore repressor activity. This prediction was confirmed by measuring the quantity of the iron–sulfur centre of NsrR to restore repressor activity.

Nitric oxide inactivation of the transcription factors NsrR and FNR by nitric oxide, and the dependence of their repair on the gonococcal YtfE orthologue, DnrN. (a) On exposure to a high concentration of NO, NsrR is inactivated in both the parent strain (open bars) and in the *dnrN* mutant (solid bars). Two hours after exposure, NsrR repression has been restored in the parent strain, but derepression of *norB* transcription is maintained in the *dnrN* mutant. The predicted nitrosation state of the iron–sulfur centre of NsrR before, 1 h after and 3 h after exposure to NO is depicted below the graph. (b) Inactivation of FNR by NO-induced damage prevents *aniA* expression immediately following exposure to NO. Unlike the parent strain (open bars) in which damage is repaired, damage in the *dnrN* mutant persists until the NO has been reduced and FNR has been replaced by *de novo* synthesis (solid bars). The figure is based upon Fig. 3 of Overton et al. (2008) (Copyright American Society for Microbiology).

To understand why bacteria maintain duplicated pathways, isogenic strains of *E. coli* were constructed that lack either the periplasmic nitrate reductase, Nap, or the cytoplasmic NarG. After laboratory growth in the presence of a high concentration of nitrate, the cytoplasmic enzyme NarG was shown to contribute the vast majority of the rate of nitrate reduction by the parent strain. A chemostat was inoculated with equal quantities of the two mutant strains to see which pathway provides a selective advantage under different growth conditions. During nitrate-limited growth, the strain expressing only the periplasmic pathway rapidly dominated the culture, but the opposite was true as soon as growth was

**Major pathways for nitrate and nitrite reduction in *E. coli***

Unlike the gonococcus, enteric bacteria are not denitrifiers. They convert nitrate via nitrite to ammonia. It was 20 years after starting work on the c-type cytochromes of *E. coli* that...
carbon-limited (Potter et al., 1999). These results were beautifully reproducible – and reversible. This was the first indication that when enteric bacteria live in the human body where energy sources are abundant but electron acceptors such as nitrate or nitrite are scarce, it is the periplasmic pathway that provides the selective advantage. Conversely, the cytoplasmic pathway provides a selective advantage for survival in wastewater treatment plants or soil, where nitrate is abundant but carbon is limiting.

**Regulation of the two pathways for nitrate reduction to ammonia**

Both of the pathways for nitrate reduction to ammonia are activated only during anaerobic growth, and this requires the intact iron–sulfur centre of the FNR protein. The key player in selecting which pathway is most active is the two-component regulatory system, NarX–NarL. Phosphorylated NarL activates the cytoplasmic pathway, but it represses synthesis of the periplasmic nitrate and nitrite reductases. It was largely the Stewart group that showed how NarX and NarL regulate nitrate reduction (Rabin & Stewart, 1993; Stewart, 1993). Steve Busby and Doug Browning have been the key collaborators in demonstrating how nitrite reduction is regulated (Browning et al., 2006, 2008). The groups of Patricia Kiley and Jeff Green demonstrated how the FNR protein uses its iron–sulfur centre to sense the presence or absence of oxygen (Khoroshilova et al., 1997; Green et al., 1996).

The dawn of the 21st century saw the rapid development of systems biology or, as some believe, spray and pray experiments. We and other laboratories were discovering many operons that are regulated by FNR, raising the question of how many different transcripts it regulates. Three points were critical for achieving an answer to this question. First, many FNR-activated genes require specific growth conditions, as were genes encoding proteins previously implicated in the response to nitrosative stress (Table 1). The breakthrough to link these gene products to nitrosative stress was made following a bioinformatic analysis by Rodionov et al. (2005), supported by experimental evidence from Bodenmiller & Spiro (2006). These groups recognized that the product of the yejB gene, which was renamed nsrR, is an orthologue of the NsrR protein from *Nitrosomonas europaea* (Beaumont et al., 2004). The NsrR designation originally signified that it was a ‘nitrite-sensitive repressor’ protein that regulates synthesis of the nitrite reductase, nirK. However, many subsequent studies with NsrR from different bacteria have established that the signal to which NsrR responds is low concentrations of nitric oxide that accumulate in the bacterial cytoplasm. Bodenmiller & Spiro (2006) showed that NsrR represses synthesis of YfeE, and provided the basis for further whole genome microarray experiments with RNA isolated from

NarL and NarP can recognize identical DNA-binding sites and that they regulate metabolic pathways that generate many secondary regulatory signals meant that the numbers of operons controlled directly by NarL and NarP could not be deduced from microarray data alone. Nevertheless, these studies with narXL and narP mutants allowed additional conclusions to be drawn. First, only a few transcripts are strongly activated by NarL phosphorylated in response to the presence of nitrate. These include genes for NarGHJI and its partner dehydrogenase, FdnGHI; *narK*, which encodes a transport protein of the major facilitator family; the *nirB* operon encoding the cytoplasmic nitrite reductase; the *ogt* gene encoding the DNA repair enzyme, O6-alkylguanine-DNA-alkyltransferase; and two genes of unknown function, *yeaR* and *youG*. Secondly, the data confirmed previous studies which showed that NarL primarily activates genes whose products are required during growth in the presence of high concentrations of nitrate, but represses transcription of genes required for growth in the absence of nitrate. In contrast, NarP appeared to activate genes required for the anaerobic utilization of low concentrations of nitrate (and to a lesser extent nitrite), but represses genes required for fermentative growth in the absence of alternative terminal electron acceptors to oxygen, nitrate or nitrite (see also Wang & Gunsalus, 2000). This therefore revealed a major role for NarP in regulating the switch between fermentation and anaerobic respiratory growth under conditions relevant to those found in the lower gastro-intestinal tract.

**Genes of unknown function expressed during nitrosative stress**

Transcription of several of the genes mentioned above was highly upregulated under conditions in which bacteria were likely to be most exposed to nitrosative stress, for example, during anaerobic growth in the presence of nitrite, but in the absence of a functional FNR protein (Constantinidou et al., 2006). Some other genes of unknown function were also induced under these conditions, as were genes encoding proteins previously implicated in the response to nitrosative stress (Table 1). The data confirmed previous studies which showed that NarL primarily activates genes whose products are required during growth in the presence of high concentrations of nitrate, but represses transcription of genes required for growth in the absence of nitrate. In contrast, NarP appeared to activate genes required for the anaerobic utilization of low concentrations of nitrate (and to a lesser extent nitrite), but represses genes required for fermentative growth in the absence of alternative terminal electron acceptors to oxygen, nitrate or nitrite (see also Wang & Gunsalus, 2000). This therefore revealed a major role for NarP in regulating the switch between fermentation and anaerobic respiratory growth under conditions relevant to those found in the lower gastro-intestinal tract.
both *N. gonorrhoeae* and *E. coli* during growth under nitrosative stress (Filenko *et al.*, 2007; Rankin *et al.*, 2008; Browning *et al.*, 2010). The NsrR from *E. coli* regulates about 20 transcripts; in the gonococcus, far fewer genes are repressed by NsrR. However, most of the genes regulated by nitrosative stress during growth in the presence of nitrate or nitrite were clearly regulated not by NarL or NarP but by NsrR (clear exceptions were the *yeaR–yoaG* operon and the *ogt* gene; Lin *et al.*, 2007; Squire *et al.*, 2009). One of the transcripts most highly regulated was the hybrid cluster protein Hcp (Chismon *et al.*, 2010). Note that expression of this gene is totally dependent upon anaerobiosis and the FNR protein (Table 1). In contrast, although expression of the bacterial flavohaemoglobin gene *hmp* was also induced under conditions of nitrosative stress, it responded in the opposite way to FNR, being repressed by FNR by Poole *et al.*, 1996; Cruz-Ramos *et al.*, 2002; Corker & Poole, 2003; Justino *et al.*, 2005; Constantinidou *et al.*, 2006; Filenko *et al.*, 2007). Transcription of *hmp* was highest in an *fnr* mutant exposed to high levels of nitrite. The same was true of transcripts of other genes of unknown function, for example, *ytfe, yeaR* and *yoaG*.

Although enteric bacteria are not denitrifiers, nevertheless, some NO is generated as a side product of nitrite reduction to ammonia (Smith, 1983). *E. coli* must also be able to protect itself against NO generated by the human host, so it has evolved protection mechanisms based upon NO removal. The iron–sulfur centre of FNR is one of the targets that can be damaged by nitric oxide. Poole and colleagues have argued that inactivation of FNR by NO is a physiologically important control mechanism, the rationale being that, in the absence of oxygen required for its NO oxygenase activity, Hmp catalyses the reduction of NO to N₂O (Cruz-Ramos *et al.*, 2002; Corker & Poole, 2003; Pullan *et al.*, 2007). However, this reduction capacity is low compared with that of NorVW or NrFA. The alternative view is therefore that FNR represses transcription of *hmp* under anaerobic conditions so that alternative, more effective, protection mechanisms are activated anaerobically (Vine & Cole, 2011a). It is possible, however, that Hmp synthesis becomes physiologically relevant under extreme environmental conditions. Derepression of Hmp synthesis during aerobic growth when FNR is inactive is entirely consistent with the well documented role for Hmp in protecting bacteria against nitrosative stress in aerobic environments, for example, in vivo in macrophages or in model systems where mammalian cells are challenged with NO in an aerobic environment (Stevanin *et al.*, 2000, 2002; Gilberthorpe & Poole, 2008; Richardson *et al.*, 2006; Svensson *et al.*, 2010, amongst many other references). Clearly, however, NsrR is the key player in the response to nitric oxide-mediated nitrosative stress.

### Table 1. Proteins implicated in the generation, removal and repair of damage caused by reactive nitrogen species

This table is based upon Table 1 from Vine & Cole (2011b) (Copyright American Society for Microbiology). (+), Transcription activation; (–), repression by the transcription factor.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Transcription regulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NarGHI</td>
<td>Cytoplasmic nitrate reduction</td>
<td>FNR (+); NarXL (+)</td>
<td>Constantinidou <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>NirBD</td>
<td>Cytoplasmic nitrite reduction</td>
<td>FNR (+); NarXL (+); NarQP (+)</td>
<td>Jackson <em>et al.</em> (1981)</td>
</tr>
<tr>
<td>NarK</td>
<td>Nitrate–nitrite transport</td>
<td>FNR (+); NarXL (+); NarQP (+)</td>
<td>Metheringham &amp; Cole (1997)</td>
</tr>
<tr>
<td>NirC</td>
<td>Nitrite transport</td>
<td>FNR (+); NarXL (+); NarQP (+)</td>
<td>Metheringham &amp; Cole (1997)</td>
</tr>
<tr>
<td>Hcp</td>
<td>Controversial, see text</td>
<td>FNR (+); Nsr (–)</td>
<td>Wolfe <em>et al.</em> (2002); Constantinidou <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>NapAB</td>
<td>Periplasmic nitrate reduction</td>
<td>FNR (+); NarXL (+); NarQP (+)</td>
<td>Page <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>NrfAB</td>
<td>Periplasmic nitrite reduction</td>
<td>FNR (+); NarXL (+); NarQP (+)</td>
<td>Squire <em>et al.</em> (2009)</td>
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<tr>
<td>Ogt</td>
<td>DNA repair</td>
<td>NarXL (+)</td>
<td>Constantinidou <em>et al.</em> (2006)</td>
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| YeaR-YoaG | Unknown | NarXL (+) | Constanti

### Unanswered – or controversial – questions concerning management of nitrosative stress in *E. coli*

Despite the extensive literature on nitrosative stress in *E. coli*, some of the simplest questions still either remain unanswered or have extremely controversial answers. First, there are few methods available to detect the accumulation of NO in the bacterial cytoplasm. One approach used to show that NO is produced by Anammox bacteria has been the use of the dye DAF2-DA (4,5-diaminofluorescein diacetate), which is freely permeable across membranes and is converted to a fluorescent derivative in the presence of NO (Kartal *et al.*, 2011). An alternative is to use an
NsR-dependent promoter linked to a reporter gene to signal NO accumulation (Hutchings et al., 2000; Cruz-Ramos et al., 2002; Bodenmiller & Spiro, 2006; Partridge et al., 2009; Tucker et al., 2008). Vine et al. (2011) have linked three NsrR-regulated promoters to the E. coli lacZ gene in the promoter-probe vector, pRW50, and used the resulting construct to detect NO generation in the E. coli cytoplasm. Assays revealed low levels of promoter activity during anaerobic growth in the absence of nitrosative stress, but activity is derepressed in an nsrR mutant. This assay was then used to show that transcription is induced far more strongly in the presence of nitrite than nitrate, confirming that nitrite must accumulate before NO is formed.

There are extensive data showing that NO is a side product formed when NarG is incubated with nitrite but in the absence of nitrate (Smith, 1983; Calmels et al., 1988; Ji & Hollocher, 1988; Ralt et al., 1988; Metheringham & Cole, 1997; Gilberthorpe & Poole, 2008). Nevertheless, others have claimed that one of the two nitrite reductases is responsible for NO synthesis (see, for example, Corker & Poole, 2003; Weiss, 2006). To resolve this controversy, the NsrR-dependent hcp promoter assay was used to study NO formation by various mutants. The results confirmed that, in the absence of the nitrate reductase, NarG, very little NO accumulates in the cytoplasm, even when a high concentration of nitrite is provided in the culture (Vine et al., 2011).

**Multiple pathways for nitric oxide reduction: chemistry, biochemistry or both?**

Many questions at different levels concerning the reduction of nitric oxide remain unanswered. NO is a free radical with a single unpaired electron; consequently, it is extremely reactive. Transfer of this unpaired electron to a metal will oxidize the metal and generate first the intermediate NO•. Conversely, transfer of an electron from a metal will oxidize the metal and generate first the intermediate NO− and then nitrous oxide, N2O.

Three enzymes are known to reduce NO to nitrous oxide or ammonia during anaerobic growth: NorVV, Hmp and NrfA (Gomes et al., 2002; Gardner & Gardner, 2002; Hausladen et al., 1998; Poock et al., 2002). There has been considerable debate about which of these proteins is most important for protecting anaerobic E. coli against nitric oxide, but this debate has been rather sterile because it has largely ignored both the conditions under which E. coli is growing and whether the source of the nitric oxide is inside or outside the cell. In denitrifying bacteria, nitric oxide reductases (NorB or NorBC) reduce NO to N2O, and in enteric bacteria, the NorVV system, known as flavohemoglobin Hmp catalyses a similar reaction. In contrast, the cytochrome c nitrite reductases, NrfAB or NrfAH, catalyse the rapid reduction of NO to ammonia (Poock et al., 2002; van Wonderen et al., 2008). Reasons for challenging the physiological significance of these reactions are that the turnover number for NO reduction by Hmp is low (Gardner & Gardner, 2002), and conversely the Km for NO reduction by NrfA is high (van Wonderen et al., 2008). Consequently, these proteins are deemed to be kinetically unsuitable for primary roles in nitric oxide removal. We recently constructed a mutant defective in both of the E. coli nitrite reductases, NrfA and NirBD, as well as Hmp and NorVV (Vine & Cole, 2011b). After growth under conditions of nitrosative stress, this mutant still reduced NO at a rate comparable to the parent strain in which all four pathways were functional. This result can be explained in two ways: either there is a major pathway for NO reduction that remains to be identified, or NO is consumed by a combination of chemical reactions with metalloproteins and other bacterial components, followed by repair of the damage by a reductive pathway. It will be necessary to determine the function of the various y-gene products induced during growth under nitrosative stress to resolve these possibilities.

**Four mechanisms to protect against nitric oxide damage: is the enigmatic hybrid cluster protein, Hcp, involved?**

The crystal structure of the hybrid cluster protein, Hcp (which was originally known as the prismane protein), revealed the presence of a novel ‘hybrid’ iron–sulfur cluster that is so far unique in biology. Instead of having a typical [4Fe–4S] cluster, it has a [4Fe–2S–2O] cluster (Arndsen et al., 1998; van den Berg et al., 2000; Wolfe et al., 2002). According to dogma, Hcp is a hydroxylamine reductase that protects bacteria against the toxicity of hydroxylamine generated during nitrite reduction to ammonia. This is based upon the ability of Hcp purified from E. coli, *Pyrococcus furiosus* and *Rhodobacter capsulatus* E1F1 to catalyse the reduction of hydroxylamine to ammonia using electrons from NADH or NADPH (Wolfe et al., 2002; Cabello et al., 2004; Overeijnder et al., 2009). However, there are many reasons why this annotation is almost certainly wrong. For example, its synthesis is not regulated in response to hydroxylamine, but by NO-dependent inactivation of repression by NsrR. Rates of hydroxylamine reduction to ammonia by an hcp–hcr deletion strain were identical to that of the parent strain, and growth inhibition of the hcp–hcr mutant was identical to that of the parent. Furthermore, the Km of Hcp for hydroxylamine reduction to ammonia at a physiological pH is three orders of magnitude higher than the concentration of hydroxylamine that would completely inhibit *E. coli* growth, explaining why the hydroxylamine reductase activity of Hcp is totally ineffective in providing protection against hydroxylamine toxicity. Finally, there are no reports of any natural environment in which the concentration of hydroxylamine approaches the Km value of Hcp for NH2OH.

To determine whether *E. coli* Hcp and its reductase, Hcr, provide the missing pathway for nitric oxide reduction, an hcp–hcr deletion mutant was shown to reduce NO as rapidly as the parent. Anaerobic growth in the presence of
nitrate or nitrite failed to reveal a clear phenotype for the mutant. In contrast, a strong phenotype was detected when the deletion mutation was transferred into the quadruple mutant that is defective in all previously known NO reductases. The quadruple mutant was able to grow well in the presence of nitrate, but the strain with all five mutations would not grow at all under these conditions. This observation, which is currently being documented more fully, strongly suggests that the hybrid cluster protein is a key player in the management of nitrosative stress. Four possible mechanisms for achieving this protection can be postulated.

First, there might be a barrier to the entry of NO into the cytoplasm or a mechanism to transport it out of the cell. Alternatively, the NO can be removed from damaged molecules and reduced to a harmless product. Thirdly, the damage might be repaired by a more elaborate excision–repair pathway as fast as it is formed. For example, a damaged iron or iron–sulfur centre might be removed enzymically before being reconstituted. A fourth possibility is that the cell might increase the rate of synthesis of the components that are most vulnerable to damage. Although we know very little about how damaged iron–sulfur centres are repaired, we propose that enteric bacteria use more than one of these mechanisms. This made us consider the functions of other proteins that are synthesized in parallel with the nitrate reductase, NarG. Surprisingly, there are very few of them, but the most notable is the hybrid cluster protein, Hcp.

Our current hypothesis is that, during the repair of nitrosative damage, an extremely toxic molecule is formed. It is the role of the hybrid cluster protein either to prevent formation of this toxic product, to replace metalloproteins damaged by nitrosation or to detoxify it as rapidly as it is formed, almost certainly by reducing it to a harmless product. Current research is directed towards resolving these three possibilities.

Whatever role is established for Hcp, the phenotype of the strain with five mutations including the \( hcp-hcr \) deletion indicates that there must be an enzyme that forms the toxic intermediate. It should be possible to use suppression genetics to identify this hypothetical enzyme, by introducing a sixth mutation into the pentuple mutant. Key candidates for this enzyme are products of the various \( y \)-genes of unknown function that are expressed in response to nitrosative stress; they include \( ygbA \), \( yeaR-yoaG \), \( yibIH \) and \( ytfE \). The last of these is especially interesting because the role of \( YtfE \) (RIC) in the repair of metalloproteins damaged by oxidative and nitrosative stress is already well documented. Does repair by \( YtfE \) involve the release of NO or a more toxic derivative of NO? If so, is it the role of Hcp–Hcr to either remove this NO (presumably by reduction) or provide an alternative pathway for damage repair? A unique structure such as the hybrid cluster of Hcp implies a unique function: this might be the resynthesis of iron–sulfur centres following damage by nitrosation. Indeed, Hcp–Hcr could even be part of a previously unreported pathway for the de novo synthesis of iron–sulfur centres during anaerobic growth. Such a pathway might also be important under other growth conditions, for example, under conditions of oxidative stress. This would be consistent with results obtained in other laboratories concerning the function of not only Hcp but also \( YtfE \) (Almeida et al., 2006; Justino et al., 2007; Overton et al., 2008).

**Summary and conclusions**

In summary, this brief survey of recent physiological studies of the gonococcus has revealed two pathways for catalysing the same process, the reduction of nitrate via nitrite to ammonia: dual two-component regulatory systems that detect and regulate the response to nitrate (and to a lesser extent, nitrite) and multiple \( y \)-gene products with functions that are clearly related, and possibly might overlap. There are many outstanding questions that emphasize the main theme of this review: an in-depth understanding of how pathogenic bacteria avoid host defences requires detailed knowledge of their physiology and biochemistry.

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