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The production and detoxification of a potent cytotoxin, nitric oxide, by pathogenic enteric bacteria

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Abstract

The nitrogen cycle is based on several redox reactions that are mainly accomplished by prokaryotic organisms, some archaea and a few eukaryotes, which use these reactions for assimilatory, dissimilatory or respiratory purposes. One group is the Enterobacteriaceae family of Gammaproteobacteria, which have their natural habitats in soil, marine environments or the intestines of humans and other warm-blooded animals. Some of the genera are pathogenic and usually associated with intestinal infections. Our body possesses several physical and chemical defence mechanisms to prevent pathogenic enteric bacteria from invading the gastrointestinal tract. One response of the innate immune system is to activate macrophages, which produce the potent cytotoxin nitric oxide (NO). However, some pathogens have evolved the ability to detoxify NO to less toxic compounds, such as the neuropharmacological agent and greenhouse gas nitrous oxide (N_2O), which enables them to overcome the host's attack. The same mechanisms may be used by bacteria producing NO endogenously as a by-product of anaerobic nitrate respiration. In the present review, we provide a brief introduction into the NO detoxification mechanisms of two members of the Enterobacteriaceae family: *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. These are discussed as comparative non-pathogenic and pathogenic model systems in order to investigate the importance of detoxifying NO and producing N_2O for the pathogenicity of enteric bacteria.

Enteric bacteria, NO and human health

Enteric bacteria find their natural habitat in the intestines of humans and other warm-blooded animals. Some of the genera are pathogenic and usually associated with intestinal infections, whereas others are essential and are part of the normal flora. Examples are the pathogenic *Salmonella*, which is a common source of food poisoning, compared with commensal non-pathogenic *Escherichia coli* strains, which have beneficial traits for humans, such as synthesizing vitamin K from undigested material in the large intestine [1]. Physical and chemical host barriers of the innate immune system generally protect the host from invading pathogens by activating macrophages, a special type of phagocyte, to engulf and destroy the invaders. Activated macrophages produce ROS (reactive oxygen species) and RNS (reactive nitrogen species), which are able to modify or inactivate proteins, lipids and nucleic acid compounds of the engulfed micro-organism, and thereby kill them [2]. One RNS that has sparked a great deal of interest in recent times is the potent cytotoxin nitric oxide (NO) that is lethal to most pathogens. NO is generated in macrophage lysozymes by iNOS (inducible nitric oxide synthase). When iNOS becomes activated, it catalyses the oxidation of L-arginine to L-citrulline and NO [3]. The generation of ROS is performed by the NADPH oxidase Phox, and genetic defects affecting this enzyme lead to an increased rate of infections in humans [4]. Phox reduces O_2 to O_2^-, which dismutates into hydrogen peroxide. The reactivity of NO and hydrogen peroxide results in the generation of other reactive compounds such as peroxynitrite. If mice lack iNOS or Phox, or both, they are much more susceptible to *Salmonella* infections, resulting in higher fatality rates and increased tissue damage of liver and spleen [5,6]. On the one hand, this underpins the importance of both enzymes for the immune system to deter invading pathogens. On the other hand, it highlights the importance of detoxification mechanisms for pathogenic bacteria such as *Salmonella*. In addition, NO is generated after a nitrate-rich meal. Dietary nitrate produces salivary nitrite, which becomes acidified in the stomach and is further converted into NO [7,8]. It has been shown that the NO levels generated in the stomach are far beyond its beneficial use as a vasodilator and that it supports the killing of pathogens in addition to the stomach acidity [7]. However, some pathogens such as *Salmonella* have evolved the ability to protect themselves against oxidative and nitrosative stresses. They are able to detoxify NO and related RNS to less toxic compounds and thereby ensure their survival. The same defence mechanisms may be used by bacteria producing NO endogenously as a

Key words: *Escherichia coli*, nitrate respiration, nitric oxide detoxification, nitrous oxide, pathogenicity, *Salmonella enterica* serovar Typhimurium.

Abbreviations used: FNR, fumarate and nitrate reductase regulator; Fur, ferric-uptake regulator; iNOS, inducible nitric oxide synthase; MetR, methionine repressor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCV, *Salmonella*-containing vacuole; SPI-2, *Salmonella* pathogenicity island 2.

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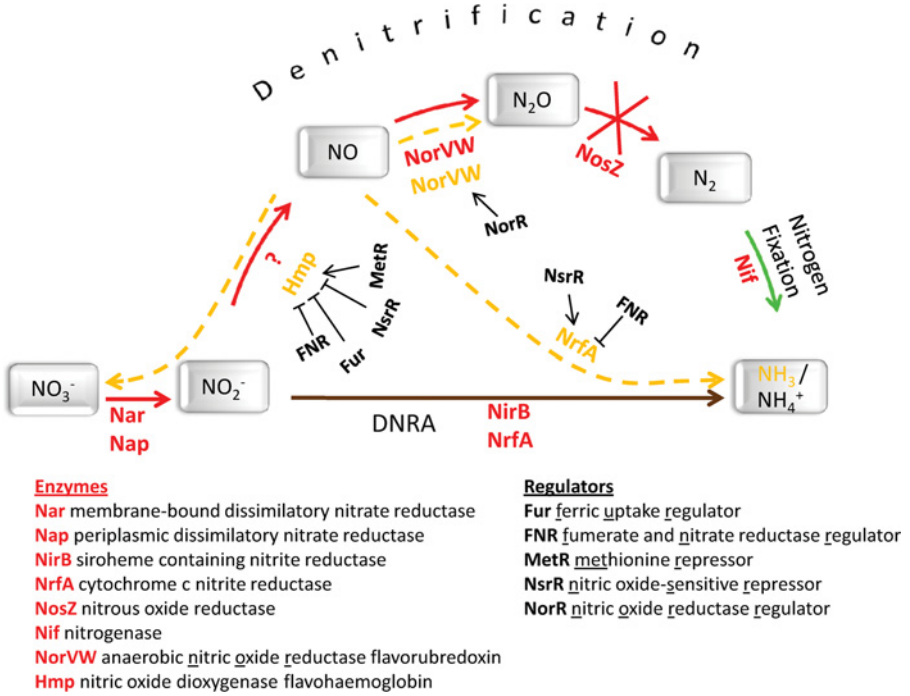
Figure 1 | **Truncated denitrification pathways in *E. coli* and *Salmonella* Typhimurium**

Nitrate respiration in *E. coli* and *Salmonella* is a truncated version of the denitrification pathway (red arrows). Unlike many soil bacteria, *E. coli* and *Salmonella* lack NosZ; indicated by a red cross. NO is a toxic intermediate. The main enzymes involved in NO detoxification alongside their regulators are shown. The NO detoxification pathways are indicated by yellow broken arrows. Enzymes involved in these pathways are shown in yellow. Positive regulation is highlighted by arrows, and negative regulation by perpendicular lines. Other enzymes are shown in red.



by-product of their own metabolism during anaerobic nitrate respiration.

NO detoxification in enteric bacteria

As *E. coli* and *Salmonella* are facultative anaerobes, O_2 is their preferred energy source, if present. However, if there is a shortage of O_2, they are able to switch to nitrate respiration to maintain their metabolism in a process called denitrification [9]. Unlike many soil bacteria, *E. coli* and *Salmonella* undergo only truncated denitrification, where the alternative electron acceptor nitrate is converted into nitrous oxide (N_2O) via nitrite and NO. The subsequent conversion of nitrous oxide into dinitrogen gas is lacking. The enzymes involved in these reactions (Figure 1) are dependent on cofactors for correct functioning, most commonly metal cofactors such as molybdenum, copper and iron–sulfur [Fe–S] clusters. The lack of N_2O reduction only makes a minor difference to the bacterium bioenergetically [9], but, on the other hand, the ability to detoxify NO is very important. Although it has been controversial for a long time whether NO itself is toxic or only the resulting RNS [10], recent studies have clearly proven that NO has cytostatic and cytotoxic effects on both aerobically and anaerobically grown cultures [11,12]. Its reactivity with [Fe–S] clusters, thiol groups and ROS results in extensive damage of DNA, proteins and transcription factors, in particular [13–15].

Enteric pathogens must have evolved mechanisms to overcome NO produced by the immune system as well as to defend themselves against their own toxic metabolites. *E. coli* and *Salmonella* are known to possess three major enzymes to perform this role. They comprise the soluble flavohaemoglobin Hmp, the di-iron-centred flavorubredoxin NorV with its NADH-dependent oxidoreductase NorW (NorVW) and the cytochrome *c* nitrite reductase NrfA [16,17]. All enzymes vary in importance under different environmental conditions. Hmp is able to cope with oxic as well as anoxic conditions, and produces nitrate and N_2O respectively. Both the NorVW and Nrf enzymes are only active under anaerobic or micro-oxic conditions [18]. NorVW reduces NO to N_2O, whereas NrfA uses either NO or nitrite to form ammonia. It has been shown that NorV and NrfA are the most important enzymes in anaerobic NO detoxification of *Salmonella* [16]. Hmp has only a minor role in NO detoxification under anoxic conditions, but it is the crucial enzyme when O_2 is present [16–18]. The combined activity of the three enzymes allows *Salmonella* and *E. coli* to be very flexible in their metabolism and hence helps them to survive in a range of different environments. This ability is also advantageous outside the host because high nitrate

concentrations and therefore high NO generation is also seen in wastewater and soil. High nitrate levels in these environments are mainly caused by manure from humans and other animals and the excessive use of nitrate-containing fertilizers.

Another reason for this high flexibility is due to various transcription factors being differently transcribed under specific conditions [19]: the main regulators that mediate a response to NO in *Salmonella* and *E. coli* include NorR, NsrR, FNR (fumarate and nitrate reductase regulator) and MetR (methionine repressor) [12].

NorR exclusively regulates the *norVW* genes in response to nitrosative stress. MetR is implicated in the regulation of *hmp* in both organisms, alongside the NO-sensitive repressor NsrR that, in addition, also regulates the expression of *nrfA* [19–21]. NsrR belongs to the Rrf2 family of transcriptional repressors and senses NO specifically by a [2Fe–2S] cluster [22]. This assumption results from great similarities between NsrR and other [2Fe–2S] cluster-containing members of the Rrf2 family such as IscR or RirA. The presence of the [Fe–S] clusters makes the protein structure and binding prone to damage by NO. It has been reported that *E. coli* genes, which are repressed by NsrR, are derepressed after exposure to NO [23]. Other regulators that are important in stress response and co-ordination of gene expression are FNR and Fur (ferric-uptake regulator) [23–25]. FNR possesses a master function in the transition between aerobic and anaerobic growth and mediates the up-regulation of several operons in response to nitrate and nitrite [25]. *Hmp* and *ytfE* are among the genes that are repressed by FNR, but the addition of either nitrite or nitrate causes an activation of the gene expression. This indicates a putative regulatory mechanism, which ensures that the expression of *hmp* will not be disabled during exposure to RNS. Exposure to NO damages the [Fe–S] clusters of FNR and results in the derepression of the protective flavohaemoglobin *hmpA* [26]. It has been demonstrated that *ytfE* plays a crucial role in the repair of NO- and ROS-damaged [Fe–S] clusters [27]. Furthermore, NO-sensitivity and growth impairment of *ytfE* mutants showed its importance in the response to oxidative and nitrosative stresses [14] and its di-iron centre has been structurally characterized [28]. Fur is also affected by the presence of NO, potentially by a reaction of the protein-bound iron with NO [23]. Fur mainly regulates genes that are involved in the uptake of iron, but it also moderately regulates *hmp* expression [12]. It has been proposed that Fur regulation becomes important once iron is limited; however, there are still controversial opinions about the repressor function of *hmp* [12].

Salmonella also utilizes SPI-2 (*Salmonella* pathogenicity island 2) for NO protection. SPI-2 encodes a TTSS (Type III secretion system), which allows formation of an SCV (*Salmonella*-containing vacuole) in the intracellular environment and prevents lysosomal fusion. This prevents the co-localization of Phox and iNOS with the SCV, hence reducing the exposure of *Salmonella* to nitrosative and oxidative stresses [29].

However, we and others believe that additional unknown pathways with important roles in NO protection remain to be characterized for both organisms [30]. In search of such mechanisms, transcriptomic analyses have proven to be helpful to highlight potential genes involved [19]. Gene annotations based on homology provide some insight into possible proteins expressed, but do not always highlight functions that are of higher physiological relevance. Therefore the function of putative NO-detoxification genes and proteins needs further investigation, particularly with respect to infection.

N_2O production in enteric bacteria

Salmonella and *E. coli* are commonly exposed to different stresses as they have various interactions with the body. This suggests that their response to stresses such as nitrosative stress and hence N_2O production varies as well. Since NO is highly reactive, it will quickly become detoxified by the conversion into nitrous oxide in the cytoplasm of *Salmonella* and *E. coli*. This process serves to convert a potent cytotoxin into a potent greenhouse gas.

Enteric bacteria can produce NO as a side product of nitrate or nitrite metabolism. One major source of this NO in *Salmonella* has been suggested to be the reduction of nitrite by the NarG nitrate reductase [31]. This endogenous NO leads to derepression of genes encoding systems that are concerned with the detoxification of NO and the repair of proteins damaged by the cytotoxin. There have been reports of nitrous oxide release by pure cultures of Enterobacteriaceae, including *E. coli*, *Klebsiella pneumoniae* and *Salmonella enterica* during nitrate metabolism that presumably reflects NO being converted into nitrous oxide [32,33]. Whether there is a physiological importance for generating and releasing the neuropharmacological agent nitrous oxide to an enteric pathogen as a side product of their nitrate metabolism has yet to be addressed, but it is an interesting question.

Concluding remarks

The significance of NO production is well studied in relation to human or murine macrophages as part of the immune defence mechanisms; however, this is not the case for the detoxification of NO and the subsequent production of N_2O by pathogens. Three enzymes have been identified that contribute significantly to NO detoxification. Therefore several questions need to be addressed in the future. Which additional mechanisms contribute to the detoxification of NO, either directly by enzymatic conversion of NO or indirectly, repairing the damage caused? Does the production of N_2O differ between pathogenic and non-pathogenic bacteria?

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References

1 Ramotar, K., Conly, J.M., Chubb, H. and Louie, T.J. (1984) Production of menaquinones by intestinal anaerobes. J. Infect. Dis. **150**, 213–218
2 Cherayil, B.J. and Antos, D. (2001) Inducible nitric oxide synthase and *Salmonella* infection. Microbes Infect. **3**, 771–776
3 Wang, Y. and Ruby, E.G. (2011) The roles of NO in microbial symbioses. Cell. Microbiol. **13**, 518–526
4 Miller, R.A. and Britigan, B.E. (1997) Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10**, 1–18
5 Shiloh, M.U., MacMicking, J.D., Nicholson, S., Brause, J.E., Potter, S. and Marino, M. (1999) Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity **10**, 29–38
6 Mastroeni, P., Vazquez-Torres, A., Fang, F.C., Xu, Y., Khan, S., Hormaeche, C.E. and Dougan, G. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. J. Exp. Med. **192**, 237–248
7 Gilchrist, M., Winyard, P.G. and Benjamin, N. (2010) Dietary nitrate: good or bad? Nitric Oxide **22**, 104–109
8 Prior, K., Hautefort, I., Hinton, J.C.D., Richardson, D.J., Rowley, G. and Robert, K.P. (2009) All stressed out: *Salmonella* pathogenesis and reactive nitrogen species. Adv. Microb. Physiol. **56**, 1–28
9 Richardson, D., Felgate, H., Watmough, N., Thomson, A. and Baggs, E. (2009) Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle: could enzymic regulation hold the key? Trends Biotechnol. **27**, 388–397
10 Brunelli, L., Crow, J.P. and Beckman, J.S. (1995) The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. Arch. Biochem. Biophys. **316**, 327–334
11 Husain, M., Bourret, T.J., McCollister, B.D., Jones-Carson, J., Laughlin, J. and Vazquez-Torres, A. (2008) Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration. J. Biol. Chem. **283**, 7682–7689
12 Spiro, S. (2007) Regulators of bacterial responses to nitric oxide. FEMS Microbiol. Rev. **31**, 193–211
13 Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S. and Keefer, L.K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science **5034**, 1001–1003
14 Justino, M.C., Almeida, C.C., Teixeira, M. and Saraiva, L.M. (2007) *Escherichia coli* di-iron YtfE protein is necessary for the repair of stress-damaged iron–sulfur clusters. J. Biol. Chem. **282**, 10352–10359
15 Tripathi, P., Tripathi, P., Kashyap, L. and Singh, V. (2007) The role of nitric oxide in inflammatory reactions. FEMS Immunol. Med. Microbiol. **51**, 443–452
16 Mills, P.C., Rowley, G., Spiro, S., Hinton, J.C.D. and Richardson, D.J. (2008) A combination of cytochrome *c* nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. Microbiology **154**, 1218–1228
17 Crawford, M.J. and Goldberg, D.E. (1998) Role for the *Salmonella* flavohemoglobin in protection from nitric oxide. J. Biol. Chem. **273**, 12543–12547
18 Gardner, A.M. and Gardner, P.R. (2002) Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*: evidence for a novel inducible anaerobic nitric oxide-scavenging activity. J. Biol. Chem. **277**, 8166–8171
19 Rodionov, D.A., Dubchak, I.L., Arkin, A.P., Alm, E.J. and Gelfand, M.S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. PLoS Comput. Biol. **1**, e55
20 van Wonderen, J.H., Burlat, B., Richardson, D.J., Cheesman, M.R. and Butt, J.N. (2008) The nitric oxide reductase activity of cytochrome *c* nitrite reductase from *Escherichia coli*. J. Biol. Chem. **283**, 9587–9594
21 Filenko, N., Spiro, S., Browning, D.F., Squire, D., Overton, T.W., Cole, J. and Constantinidou, C. (2007) The NsrR regulon of *Escherichia coli* K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. J. Bacteriol. **189**, 4410–4417
22 Bodenmiller, D.M. and Spiro, S. (2006) The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. J. Bacteriol. **188**, 874–881
23 Pullan, S.T., Gidley, M.D., Jones, R.A., Barrett, J., Stevanin, T.M., Read, R.C., Green, J. and Poole, R.K. (2007) Nitric oxide in chemostat-cultured *Escherichia coli* is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. J. Bacteriol. **189**, 1845–1855
24 Overton, T.W., Griffiths, L., Patel, M.D., Hobman, J.L., Penn, C.W., Cole, J.A. and Constantinidou, C. (2006) Microarray analysis of gene regulation by oxygen, nitrate, nitrite, FNR, NarL and NarP during anaerobic growth of *Escherichia coli*: new insights into microbial physiology. Biochem. Soc. Trans. **34**, 104–107
25 Constantinidou, C., Hobman, J.L., Griffiths, L., Patel, M.D., Penn, C.W., Cole, J.A. and Overton, T.W. (2006) A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as *Escherichia coli* K12 adapts from aerobic to anaerobic growth. J. Biol. Chem. **281**, 4802–4815
26 Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M.N., Scott, C., Thomson, A.J., Green, J. and Poole, R.K. (2002) NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J. **21**, 3235–3244
27 Vine, C.E., Justino, M.C., Saraiva, L.M. and Cole, J. (2010) Detection by whole genome microarrays of a spontaneous 126-gene deletion during construction of a *ytfE* mutant: confirmation that a *ytfE* mutation results in loss of repair of iron–sulfur centres in proteins damaged by oxidative or nitrosative stress. J. Microbiol. Methods **81**, 77–79
28 Todorovic, S., Justino, M.C., Wellenreuther, G., Hildebrandt, P., Murgida, D.H., Meyer-Klaucke, W. and Saraiva, L.M. (2008) Iron–sulfur repair YtfE protein from *Escherichia coli*: structural characterization of the di-iron center. J. Biol. Inorg. Chem. **13**, 765–770
29 Chakravortty, D., Hansen-Wester, I. and Hensel, M. (2002) *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. J. Exp. Med. **195**, 1155–1166
30 Vine, C.E. and Cole, J.A. (2011) Nitrosative stress in *Escherichia coli*: reduction of nitric oxide. Biochem. Soc. Trans. **39**, 213–215
31 Gilberthorpe, N.J. and Poole, R.K. (2008) Nitric oxide homeostasis in *Salmonella* Typhimurium: roles of respiratory nitrate reductase and flavohemoglobin. J. Biol. Chem. **283**, 11146–11154
32 Smith, M.S. (1983) Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. Appl. Environ. Microbiol. **45**, 1545–1547
33 Bleakley, B.H. and Tiedje, J.M. (1982) Nitrous oxide production by organisms other than nitrifiers or denitrifiers. Appl. Environ. Microbiol. **44**, 1342–1348

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