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Deciphering nitric oxide stress in bacteria with quantitative modeling

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Many pathogens depend on nitric oxide ($NO^{\bullet}$) detoxification and repair to establish an infection, and inhibitors of these systems are under investigation as next-generation antibiotics. Because of the broad reactivity of $NO^{\bullet}$ and its derivatives with biomolecules, a deep understanding of how pathogens sense and respond to $NO^{\bullet}$, as an integrated system, has been elusive. Quantitative kinetic modeling has been proposed as a method to enhance analysis and understanding of $NO^{\bullet}$ stress at the systems-level. Here we review the motivation for, current state of, and future prospects of quantitative modeling of $NO^{\bullet}$ stress in bacteria, and suggest that such mathematical approaches would prove equally useful in the study of other broadly reactive antimicrobials, such as hydrogen peroxide (H_2O_2).

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Introduction

$NO^{\bullet}$ is a potent antimicrobial produced by immune cells to combat pathogens [1••,2]. The importance of $NO^{\bullet}$ to immunity is evidenced by the many pathogens, including *Mycobacterium tuberculosis*, *Neisseria meningitides*, *Vibrio cholerae*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and enterohemorrhagic *Escherichia coli* (EHEC), whose virulence depends on $NO^{\bullet}$ detoxification and repair systems (Table 1) [3••,4–8]. Collectively, these studies suggest that knowledge of how pathogens sense and respond to $NO^{\bullet}$ could illuminate antibacterial strategies that synergize with host immunity. Research on $NO^{\bullet}$ stress has continued for over two decades, and the cumulative picture that has emerged is immensely complex [1••,9•,10–12]. This derives from the broad reactivity of $NO^{\bullet}$ and its reactive intermediates (reactive nitrogen species: RNS) with biomolecules [1••,9•,12]. Depending on the environment, dosage, and delivery rate, $NO^{\bullet}$ will destroy iron–sulfur (Fe–S) clusters, reversibly bind heme, directly react with O_2 and superoxide ($O_2^{\bullet-}$), and/or be enzymatically detoxified, whereas its derivatives ($NO_2^{\bullet}$, N_2O_3, N_2O_4, HNO, and $ONOO^-$) damage thiols, tyrosine residues, and DNA bases (Figure 1) [1••,9•,12–14]. This systems-level stress becomes even further complicated when one considers that Fe–S clusters and thiols are used for a broad range of enzymatic and regulatory functions throughout the cellular network [15,16,17•,18,19]. To decipher this response and understand how bacteria, as an integrated system, sense and respond to $NO^{\bullet}$, a quantitative understanding of intracellular $NO^{\bullet}$ reactivity is required. $NO^{\bullet}$ has many available reaction paths upon entering a cell, and the biological outcome of $NO^{\bullet}$ exposure, whether it is continued growth, bacteriostasis, expression of virulence factors, transition to an antibiotic-tolerant state and/or cell death [17•,20–22], is governed by a complex, kinetic competition. Quantitative knowledge of this competition and the factors that control it will reveal novel targets within the $NO^{\bullet}$ response network for the discovery and development of therapeutics that synergize with host-derived $NO^{\bullet}$.

Because of the complexity of the competition for $NO^{\bullet}$ among biomolecules, mathematical models are required to quantitatively analyze and understand data from $NO^{\bullet}$-stressed environments [13,14,23,24••]. Beyond data interpretation, these models enable identification of emergent properties of the $NO^{\bullet}$ response network and formulation of testable predictions concerning the impact of genetic and environmental perturbations. Here, we summarize evidence that suggests quantitative modeling will facilitate the discovery and development of $NO^{\bullet}$-based antibiotics, review the current state of $NO^{\bullet}$ models along with their contributions to the present understanding of $NO^{\bullet}$ stress, and reflect upon the future prospects of quantitative modeling to enhance the study of systems-level stresses from not only $NO^{\bullet}$, but other broadly reactive antimicrobials as well, such as H_2O_2.

$NO^{\bullet}$ detoxification and repair systems are prevalent virulence factors

The ability to withstand $NO^{\bullet}$ stress has been linked to the virulence of an impressive number of pathogens, several of which are presented in Table 1. Notably, in *S.* Typhimurium, Stevanin and colleagues found that a mutant defective in $NO^{\bullet}$ dioxygenase (Δ*hmp*) exhibited reduced survival in human macrophages, and that the effect was eliminated upon treatment with an inhibitor of

Table 1

Pathogens for which NO• detoxification or repair has been identified as a virulence factor

Pathogen	Gene(s)	Description	Ref
E. coli (enterohemorrhagic)	*norV*	Strains harboring an inactive *norV* gene (*norVs*) exhibited reduced survival in murine macrophages.	[4]
E. coli (uropathogenic)	*hmp*	Isolates from patients with urinary tract infection had increased *hmp* expression, and Δ*hmp* mutants were outcompeted by the wild-type in a mouse infection model.	[78]
M. tuberculosis	*mpa*, *pafA*, *uvrB*, *dlaT*	Mutants deficient in proteasome components (*mpa* or *pafA*) [3••] or nucleotide excision repair (*uvrB*) [31] exhibited attenuated virulence in mice.	[3••,31]
N. meningitides	*cycP*, *norB*	Mutants lacking cytochrome *c′* (*cycP*) or NO• reductase (*norB*) exhibited reduced survival in human macrophages and human nasopharyngeal mucosa organ cultures.	[6]
P. aeruginosa	*norCBD*	A mutant deficient in NO• reductase (*norCBD*) exhibited reduced viability in murine macrophages.	[8]
S. Typhimurium	*hmp*, *xth*, *nfo*, *ytfE*	Mutants lacking *hmp* exhibited reduced survival in human macrophages [25] and attenuated virulence in mice [26]. Mutations in base excision repair (*xth* and *nfo*) [32•] and Fe–S assembly (*ytfE*) [7] both caused attenuated virulence in mice.	[7,25,26,32•]
S. aureus	*hmp*	Mutants deficient in *hmp* exhibited attenuated virulence in mice.	[79]
V. cholerae	*hmpA*	Mutants lacking *hmpA* were outcompeted in a mouse intestine colonization assay.	[5]
Y. pestis	*hmp*	A mutation in *hmp* resulted in longer incubation times and attenuated virulence in rats.	[30]

inducible nitric oxide synthase (iNOS) [25]. More recently, this effect was corroborated *in vivo*, where Δ*hmp S.* Typhimurium had attenuated virulence in mice, and iNOS inhibition restored virulence [26]. In EHEC, a genomic study of clinical isolates found that the presence of a functional *norV* gene, which encodes NO• reductase, correlated with an increased frequency of hemolytic-uremic syndrome (HUS) [27]. This connection was substantiated by a study demonstrating that EHEC possessing an inactive *norV* gene exhibited reduced survival in mouse macrophages compared to those with an active *norV* [4]. Recently, the genome of the EHEC strain responsible for the 2011 outbreak in Germany, which resulted in the highest incidence of HUS on record [28], was found to contain a functional *norV* [29], lending even further support for the previous genomic study. For *Yersinia pestis*, a microarray analysis of a model rat infection identified *hmp* expression to be significantly up-regulated, and subsequent experiments revealed that a Δ*hmp* mutant exhibited attenuated virulence [30]. Beyond NO• detoxification, microbial repair systems have also been found to be important for resisting NO• stress and were shown to contribute to virulence. A transposon screen in *M. tuberculosis* found that mutations in proteasome components (*mpa* and *pafA*) and a nucleotide excision repair gene (*uvrB*) increased killing by NO• produced from acidified nitrite *in vitro*, and reduced virulence in mouse infection models [3••,31]. In *S.* Typhimurium, Richardson and colleagues found that base excision repair mutants (Δ*xth*Δ*nfo*) had attenuated virulence in mice, which was fully restored upon administration of an iNOS inhibitor [32•]. These and related studies support a role for NO• and its derivatives as key mediators of host defense, and suggest that targeting the NO• defenses of pathogens may be an effective way to inhibit infection [33].

Therapeutic potential of NO•-based antibiotics

Several studies have found chemical inhibitors of the NO• response network that increase the sensitivity of pathogens to NO• [3••,33,34••]. Two chemical inhibitors were shown to block activity of the *M. tuberculosis* proteasome, and successfully reproduced the NO•-sensitive phenotype of proteasome-deficient mutants [3••]. Helmick and colleagues found that imidazoles could inhibit NO• dioxygenase *in vitro*, and whole-cell NO• detoxification in *Staphylococcus aureus* and *E. coli* cultures, though the effects were far less pronounced in *E. coli* due to the poor Gram-negative membrane permeability of imidazoles [33]. By performing a screen to identify inhibitors of DlaT, an enzyme important for *M. tuberculosis* to tolerate NO•-stress, Bryk and colleagues discovered that rhodanines enhance killing of non-replicating *M. tuberculosis* treated with NO• by several orders of magnitude [34••]. Further, D157070 (DlaT inhibitor) reduced *M. tuberculosis* viability in murine bone-marrow macrophages. These studies demonstrate the potential of targeting the NO• response network for the discovery of novel antibiotics, and suggest that a deeper understanding of NO• stress will reveal additional therapeutic strategies for investigation, since all targets are not equally accessible, as demonstrated with imidazoles and *E. coli* [33]. It is also worth noting that, in addition to potentiating immune-derived NO•, chemicals that target the NO• response network would prove useful for therapies that directly administer exogenous NO• to infection sites. Direct administration techniques have been garnering attention in recent years, due to the ability of NO• to