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Starved *Escherichia coli* preserve reducing power under nitric oxide stress

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ABSTRACT

Nitric oxide (NO) detoxification enzymes, such as NO dioxygenase (NOD) and NO reductase (NOR), are important to the virulence of numerous bacteria. Pathogens use these defense systems to ward off immune-generated NO, and they do so in environments that contain additional stressors, such as reactive oxygen species, nutrient deprivation, and acid stress. NOD and NOR both use reducing equivalents to metabolically deactivate NO, which suggests that nutrient deprivation could negatively impact their functionality. To explore the relationship between NO detoxification and nutrient deprivation, we examined the ability of *Escherichia coli* to detoxify NO under different levels of carbon source availability in aerobic cultures. We observed failure of NO detoxification under both carbon source limitation and starvation, and those failures could have arisen from inabilities to synthesize Hmp (NOD of *E. coli*) and/or supply it with sufficient NADH (preferred electron donor). We found that when limited quantities of carbon source were provided, NO detoxification failed due to insufficient NADH, whereas starvation prevented Hmp synthesis, which enabled cells to maintain their NADH levels. This maintenance of NADH levels under starvation was confirmed to be dependent on the absence of Hmp. Intriguingly, these data show that under NO stress, carbon-starved *E. coli* are better positioned with regard to reducing power to cope with other stresses than cells that had consumed an exhaustible amount of carbon.

1. Introduction

Antibiotics are a pillar of modern medicine for the treatment of bacterial infections. With the ever increasing rise in antibiotic resistance and a comparatively weak pipeline of novel agents, there is significant demand for novel anti-infective therapies [1]. Anti-virulence therapies, which are those that disrupt host-pathogen interactions such as adherence, quorum sensing, or susceptibility to immune attack, comprise a new class of anti-infectives. These therapies would have the advantage of confining selective pressure to within the host and avoiding the destruction of commensal flora [2].

One virulence system used by a variety of bacteria is NO detoxification, because NO is utilized as an antimicrobial by different cells of the innate immune response. The importance of NO to immunity has been demonstrated in mice devoid of inducible NO synthase (iNOS), where susceptibility to infections by *Mycobacterium tuberculosis* [3] and *Salmonella enterica* [4] were increased compared to iNOS proficient controls. Further, it has been shown that numerous bacterial species require NO defense systems to reach their full pathogenic potential [5]. For example, Shimizu and colleagues investigated the importance of *norV*, which encodes an NO reductase (NOR), to enterohaemorrhagic *Escherichia coli* (EHEC) pathogenicity, and found that a functional *norV* conferred increased survival and increased capacity to produce Shiga toxin in macrophages compared to a catalytically inactive control [6]. With uropathogenic *E. coli* (UPEC), the expression of *hmp*, which encodes an NO dioxygenase (NOD), was found to be higher in patients with urinary tract infections (UTI) than an *in vitro* control exposed to urine of healthy patients [7]. Further, it was found that a Δ*hmp* UPEC strain was out-competed by wild-type cells in a murine UTI model [7]. The examples above illustrate that NOD and NOR are prominent bacterial virulence systems, and since nutrient

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deprivation is a tactic used by immune cells to neutralize pathogens [8], it is of interest to understand how nutrient availability affects these enzymes.

In this study, we focused on carbon source deprivation and two of its major consequences, reduced protein synthesis and an impaired ability to produce NADH [9]. These are important to NO detoxification, because in *E. coli* both NOD (Hmp) and NOR (NorV) are synthesized in response to NO stress [10,11], and both use reducing equivalents to metabolically inactivate NO [11,12]. Here we analyzed aerobic NO detoxification, and under such conditions an interesting network architecture emerges where Hmp and the electron transport chain (ETC) compete for O_2 and NADH (NADH is preferred reductant of Hmp [13]), whereas NO inhibits the ETC and is processed by Hmp (Fig. 1A). In this system, NO detoxification forms a 4-input AND gate (NO, O_2, NADH, Hmp) (Fig. 1B), of which carbon source deprivation can perturb two of the inputs (NADH, Hmp). Therefore, NO detoxification could fail under carbon source deprivation due to three distinct states, insufficient NADH and sufficient Hmp (01 state: NADH−, Hmp+), sufficient NADH and insufficient Hmp (10 state: NADH+, Hmp−), and insufficient levels of both NADH and Hmp (00 state: NADH−, Hmp−) (Fig. 1C). It is not obvious which will occur since NO inhibits the most significant NADH consumption pathway in aerobic *E. coli*, the ETC, and translation is reduced but not eliminated under carbon source starvation [14]. To examine these possibilities, we investigated *E. coli* NO detoxification in minimal media with various levels of glucose as the sole carbon source (replete: 2 mM, limited: 80 μM; starved: no glucose). We observed failure of NO detoxification under limited and starved conditions, where a 01 (NADH−, Hmp+) failure was present under limited conditions, and a 00 (NADH−, Hmp−) failure occurred under starved conditions. However, despite limited and starved scenarios producing states where NADH levels could not support NO detoxification, cells under starved conditions retained significantly higher NADH levels, and these levels were maintained throughout the experiment. This suggested that the inability of starved cultures to accumulate Hmp protected their NADH pools, which was confirmed with experiments where Hmp was synthesized prior to NO stress. Interestingly, the data presented here suggests that if *E. coli* NO detoxification fails due to carbon-source availability, it is better to be starved than be provided with an exhaustible substrate in terms of the NADH state of the cell.

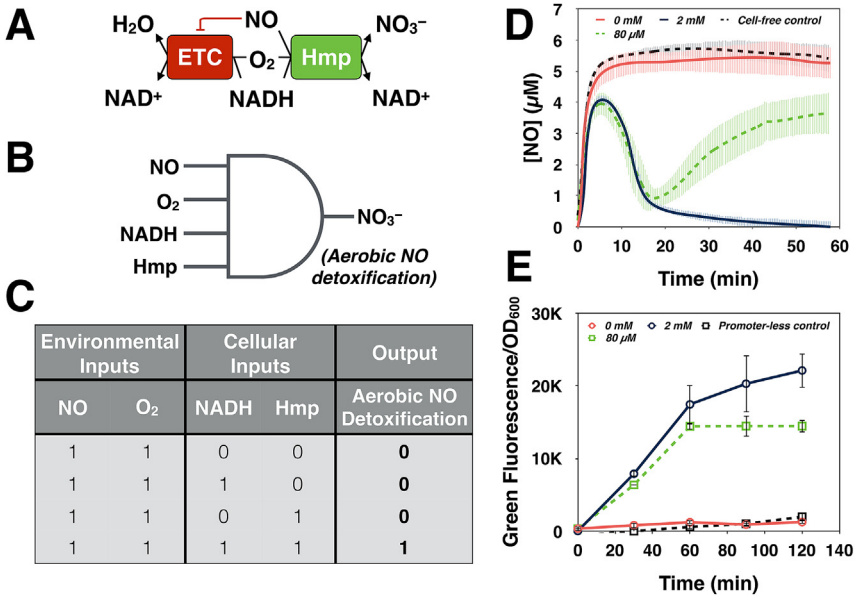
2. Materials and methods

2.1. Bacterial strains and plasmids

All strains used were derived from *E. coli* K-12 MG1655. A Δ*hmp* mutant was generated by transferring the mutation from the Keio collection [15] into MG1655 using P1-phage transduction [16]. Plasmids used in this study were derived from pUA66 [17] as further described in the Supplementary materials and methods.

2.2. Chemicals, media, and culturing conditions

DPTA NONOate (DPTA) was the NO donor (Cayman Chemicals). The bioreactor setup consisted of a 50 mL disposable conical tube, with 10 mL media open to ambient air, stirring in a stirred 37 °C water bath [18]. To prepare cultures, 1 mL of LB media was inoculated with a frozen cell stock and grown for 4 h at 37 °C with shaking (250 rpm). This was followed by a 1:100 inoculation into 1 mL of fresh M9-minimal glucose (10 mM) media, which was grown for 16 h (37 °C, 250 rpm shaking). This overnight was used to inoculate 20 mL of M9-minimal glucose (2 mM) media in a 250 mL baffled flask to a final OD_{600} of 0.01 and grown (37 °C, 250 rpm shaking) until OD_{600} = 0.2. Then, 8 mL of culture was centrifuged (21130 × g for 3 min), supernatant removed, and pellet resuspended in M9-minimal glucose media (2 mM, 80 μM, or 0 mM glucose corresponding to the bioreactor concentration). Where indicated, a second centrifugation, supernatant removal, and resuspension step was performed. Cultures were added to bioreactors to achieve a final OD_{600} of 0.05 and dosed with DPTA (100 μM, unless otherwise stated) after 10 min. Variations on this



Environmental Inputs		Cellular Inputs		Output
NO	O_2	NADH	Hmp	Aerobic NO Detoxification
1	1	0	0	**0**
1	1	1	0	**0**
1	1	0	1	**0**
1	1	1	1	**1**

Fig. 1. NO detoxification under aerobic conditions. (A) Biochemical reaction network depicting the relationship between NO, O_2, NADH, Hmp, and the ETC. (B) Aerobic NO detoxification in *E. coli* depicted as a 4-input AND gate. (C) Abridged truth table of AND gate for when environmental inputs (NO, O_2) are consistently present. (D) [NO] following the addition of 100 μM of DPTA at time 0 in *E. coli* cultures with different initial concentrations of glucose as the sole carbon source. (E) Protein production from P_{hmp} following treatment with 200 μM DPTA at time 0. Measurements were performed with Δ*hmp* harbouring a pUA66-P_{hmp}-*gfp* reporter plasmid, except for the promoter-less control, which was Δ*hmp* with pUA66. Data are the mean of 3 or more replicates, and error bars indicate ± the standard error of the mean (SEM).

procedure are outlined in the Supplementary materials and methods.

2.3. OD_{600} and fluorescence measurements

Cell concentration (optical density at 600 nm; OD_{600}) and GFP fluorescence (485/515 nm excitation/emission wavelengths) were measured on a BioTek Synergy H1 Hybrid Reader plate reader.

2.4. Biochemical measurements

NO was measured using an NO probe, ISO-NOP 2 mm NO sensor (World Precision Instruments), glucose and NADH were measured using an Amplex Red Glucose Oxidase enzymatic assay (Thermo Fisher Scientific), and an EnzyChrom NAD/NADH Assay Kit (Bio-Assays Systems), respectively. Further details are provided in the Supplementary materials and methods.

3. Results

3.1. Carbon source starvation impairs aerobic NO detoxification

To assess the impact of carbon source deprivation on NO detoxification, we measured NO concentrations ([NO]) in *E. coli* cultures treated with DPTA in M9 minimal media without a carbon source (starved) and with 80 μM (limited) or 2 mM (replete) glucose as the sole carbon source. We note that for all conditions, cells were collected from exponentially-growing populations cultured in M9 minimal media with 2 mM glucose. As depicted in Fig. 1D, under replete conditions *E. coli* was able to rapidly detoxify NO compared to the cell-free control, which indicated that Hmp was effectively synthesized and fueled with NADH (11 state: NADH+, Hmp+). We note that in previous work under comparable conditions (aerobic, minimal glucose media, $OD_{600} = 0.05$, DPTA as the NO donor), >99.8% of the cellular contribution to NO detoxification was through Hmp [18]. Under limited glucose availability (80 μM initially present), NO detoxification mirrors that of the replete condition, which indicated an 11 state, until ~20 min, when failure occurred. Under carbon source starvation, NO detoxification failed at the onset and closely followed the cell-free control.

3.2. Failure of NO detoxification under carbon source limitation coincides with glucose exhaustion

We suspected that NO detoxification ceased under carbon source limitation due to glucose exhaustion, and therefore measured glucose in replete, limited, and starved conditions after DPTA treatment. Glucose concentrations in the replete and starved samples remained constant at approximately 2 mM and undetectable, respectively, whereas under limited conditions, the concentration fell from ~40 μM at time 0 to undetectable at 20 min (Supplementary Fig. 1). We note that the initial concentration of glucose in the limited condition was 80 μM (at the time of inoculation) and that the cells consumed half of that amount within the 10 min they were present in the media prior to DPTA treatment. These data demonstrate that glucose exhaustion coincided with failure of NO detoxification in the carbon source limited samples. The rapid cessation of NO detoxification under limited conditions suggested that the culture transitioned from an 11 to a 01 state (NADH failure) because metabolite levels can change on the order of seconds or less [19], whereas the typical time-scales of protein degradation are far longer ($t_{1/2}$~7 h [20]). However, the type of failure present under starvation conditions could have originated from deficiencies in NADH (01), Hmp (10), or both (00).

3.3. Hmp is synthesized under carbon source limitation, but not under starvation

NsrR is a transcriptional repressor that is the major regulator of *hmp* expression [21]. In the absence of NO, NsrR binds the *hmp* promoter, P_{hmp}, and limits Hmp abundance to trace levels [22]. Upon exposure to NO, NsrR is deactivated resulting in synthesis of Hmp [22]. To assess the impact of carbon source availability on the ability of P_{hmp} to drive protein synthesis, we employed a P_{hmp}-*gfp* reporter in a Δ*hmp* strain. We note that Δ*hmp* cannot detoxify measurable amounts of NO under aerobic conditions, and thus we used it in this assay to obtain identical [NO] dynamics under carbon replete, limited, and starvation conditions. As illustrated in Fig. 1E, GFP was produced under replete and limited conditions, whereas GFP was essentially undetectable under starvation conditions. This data demonstrated that starvation prevented P_{hmp}-dependent protein synthesis under NO stress, which suggested that an Hmp deficiency was a component of NO detoxification failure under carbon source starvation. In addition, these data in conjunction with that in Fig. 1D provide evidence that Hmp is synthesized to sufficient levels to support NO detoxification under carbon-limited conditions.

3.4. NADH depletion underlies failure of NO detoxification under carbon source limitation

To provide additional support that NO detoxification failed under carbon source limitation due to insufficient NADH following glucose exhaustion, we quantified NADH levels in carbon-limited cultures under NO stress. Fig. 2A shows that, under carbon-limited conditions, intracellular NADH levels dropped from their value just prior to NO stress to undetectable levels at 45 min post-treatment, which was after NO detoxification had failed (Fig. 1D). We note that for replete conditions the NADH levels prior to NO stress, and 45 min after, were abundant (Supplementary Fig. 2A). These data are consistent with a 01 (NADH−, Hmp+) failure state under carbon source limitation. To show conclusively that NO detoxification ceased under those conditions due to an inability to fuel Hmp, we added chloramphenicol (CAM) and glucose (2 mM) to those cultures after failure of NO detoxification had been observed. CAM, at 100 μg/mL, prevents further protein synthesis [23], whereas the addition of glucose provided a substrate for NADH regeneration. Treatment with CAM and glucose thus facilitates a transition from a 01 to 11 state, whereas if 10 and 00 states were present the addition of CAM and glucose would not restore NO detoxification. Fig. 2B demonstrates that carbon-limited cultures exhibited a 01 failure state upon glucose exhaustion, because they were able to resume NO detoxification in the presence of CAM and glucose.

3.5. Starvation preserves NADH levels under NO stress

The data presented in Fig. 1E suggested that starvation inhibits Hmp synthesis, which would be consistent with either the 10 or 00 failure states. Conceivably, either of these scenarios was possible. Under aerobic conditions, NADH dehydrogenases are the largest consumers of NADH [24], passing the electrons on to the ETC. When starved of a carbon source, respiration slows and cellular NADH levels drop [9], reflecting conditions where the rate of NADH consumption outpaces the rate of production until a lower steady-state level is achieved. Therefore, it would be reasonable to hypothesize that carbon source starvation produced a 00 failure state. However, NO inhibits the ETC through its inhibition of the cytochrome ubiquinol terminal oxidases [25,26]. This would impair the major NADH consumption pathway, and potentially preserve NADH levels

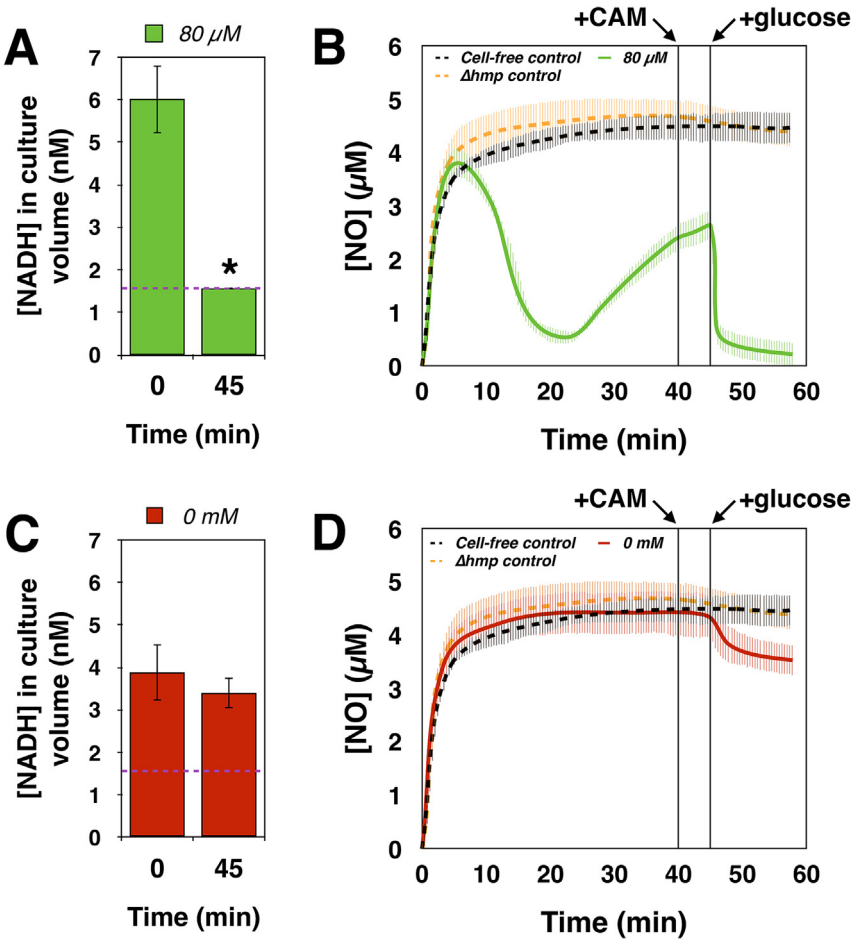


Fig. 2. Quantification of NADH levels and assessment of their impacts on NO detoxification. (A) [NADH] under carbon-limited conditions immediately before and 45 min after treatment with 100 μM of DPTA. (B) Effect of CAM and glucose addition on NO detoxification under carbon-limited conditions following treatment with 100 μM of DPTA at time 0. (C) and (D) analogous to (A) and (B) except under carbon-starvation conditions. Dashed line in (A) and (C) depicts limit of detection for NADH assay. Cell-free and Δ*hmp* controls were performed with M9 minimal 2 mM glucose media. Data are the mean of 3 replicates, and error bars indicate ± SEM. (*) indicates measurements at or below the limit of detection.

because the reduced consumption may not exceed generation from endogenous sources (*e.g.,* phospholipids [23,27]). Although Hmp could deplete NADH despite an impaired ETC, starvation impairs Hmp synthesis, and thus Hmp would be present at only trace levels. Such a scenario could produce a 10 (NADH+, Hmp−) failure state under starvation, rather than a 00 state.

To resolve whether starvation produced a 00 or 10 state, we measured NADH in starved cultures immediately before NO exposure and 45 min after. Interestingly, we observed that the level of NADH was preserved (Fig. 2C). NADH in carbon-starved cultures began at somewhat lower values, which was likely due to the 10 min incubation period prior to NO treatment, but its abundance was maintained, whereas NADH in carbon-limited cultures became undetectable. While these data established that starvation had a protective effect on NADH levels under NO stress, the question of whether starvation produced a 10 or 00 failure remained unanswered. If starvation resulted in a 10 failure the trace Hmp that was present prior to NO stress would actively detoxify NO at its full capacity, whereas for a 00 failure the trace Hmp would be limited by NADH availability. Therefore, we added CAM and glucose to starved cultures under NO stress and monitored [NO]. Fig. 2D shows that under starvation, the trace Hmp present was not functioning at its full capacity, which demonstrated that starvation produced a 00 failure where NO detoxification was impaired by both insufficient Hmp and insufficient NADH supply.

3.6. Pre-expression of Hmp depletes NADH under carbon source starvation

Intrigued by the preservation of NADH under starvation conditions and dramatic decrease in NADH upon glucose exhaustion under carbon source limitation, we hypothesized that it was the inability of starved cells to induce Hmp synthesis that allowed them to maintain their NADH levels. To test this, we employed an Hmp-GFP translational fusion [28], pre-expressed it prior to NO exposure, and delivered those cells to media that contained NO, CAM, and either replete, limited, or starved concentrations of glucose. As depicted in Fig. 3A, replete conditions supported NO detoxification, limited conditions supported detoxification for a short period of time and then failed, whereas starved cells failed at the onset. We note that the inducer-less control depicts the level of NO detoxification that can be supported by trace Hmp-GFP that accumulated in cells due to leaky expression. When NADH was quantified, we observed that starved cells had depleted their NADH to undetectable levels by 45 min, and that NADH in cultures with limited glucose had also begun to deplete their NADH by that time. As a control the replete condition showed abundant NADH at both 0 and 45 min (Supplementary Fig. 2B). These data show that NADH

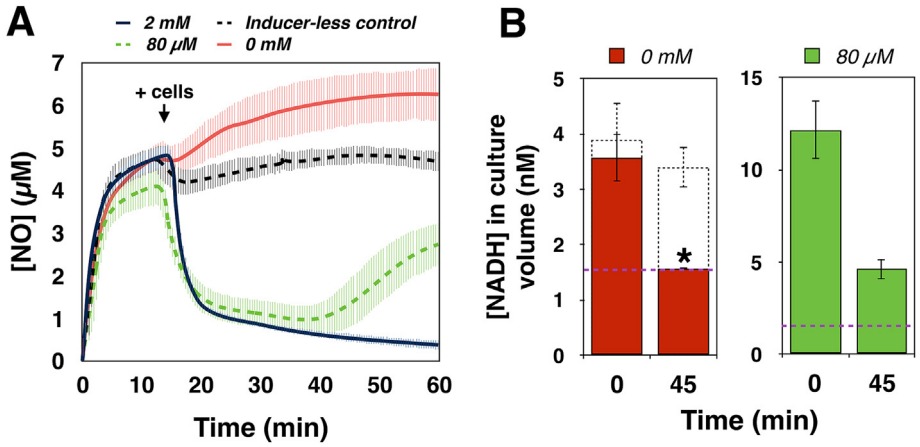


Fig. 3. NO detoxification with pre-expressed Hmp. (A) [NO] following 100 μM DPTA treatment to cultures with pre-expressed Hmp-GFP in media with different initial concentrations of glucose as the sole carbon source. Cells were added approximately 15 min after DPTA, and Hmp-GFP levels at inoculation are provided in Supplementary Fig. 3A. Inducer-free control corresponds to cells that were introduced into media with 2 mM glucose, but not induced with IPTG. (B) NADH was quantified just prior to addition of cells to NO-containing media (0 min), and 45 min later. Data are the mean of 3 replicates, and error bars indicate ± SEM. (*) indicates measurements at or below the limit of detection. For comparison, [NADH] for the starved condition without pre-expression are shown with dashed black bars in (B).

could not be preserved in NO-stressed, carbon-starved cultures when sufficient Hmp was present for detoxification.

4. Discussion

NO detoxification enzymes are important to the virulence of diverse pathogens [5], and the activities of these enzymes are directly linked to metabolism through their use of reducing equivalents for catalysis [11,12,29,30]. NO exposure can occur concurrently with nutrient deprivation, such as within the phagosomes of immune cells [8,31], which suggests that knowledge of the interplay between these two stressors is valuable. Here we examined how carbon source availability influenced NO detoxification by Hmp in aerobic *E. coli* cultures, and a model summarizing our results has been provided in Fig. 4. We characterized NO detoxification failure in environments without a carbon source (starved) and those with an exhaustible amount of carbon (limited), and discovered that carbon-starved cultures, but not carbon-limited cultures, were able to preserve their NADH levels under NO stress (Fig. 2). This effect was dependent on the inability of carbon-starved cultures to synthesize Hmp, which when combined with the inhibition of respiration by NO, reduced NADH consumption such that endogenous generation could keep pace with usage. These data suggest that *E. coli* are better off with regard to reducing power when starved of carbon during NO stress compared to when they are provided with an exhaustible amount. In consideration that bacteria employ defenses that utilize reducing equivalents for other stresses, such as alkyl hydroperoxidase which is present during normal growth and consumes NADH to detoxify H_2O_2 [9], higher NADH levels could be important for *E. coli* to cope with stresses that occur along with NO and nutrient deprivation.

The link between bacterial NO tolerance and metabolism has been strengthened in recent years. In *E. coli*, it has been shown that dihydroxyacid dehydratase, which is an enzyme in branched chain amino acid biosynthesis, is a primary target of NO that can underlie NO-induced bacteriostasis [32]. A metabolomic study of NO-stressed *Vibrio cholerae* uncovered a wide-range of metabolite perturbations that ranged from glycolysis and the TCA cycle to amino acid biosynthesis [33]. Under NO stress, *S. aureus* initiates lactate production in order to generate ATP in a redox-balanced

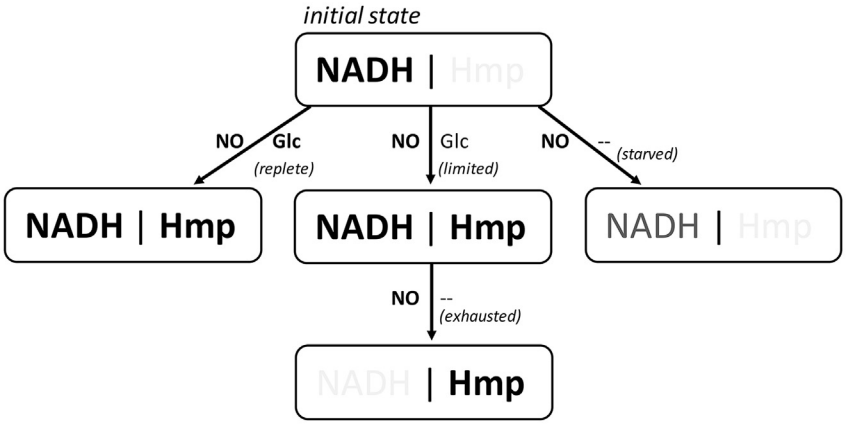


Fig. 4. Impacts of carbon source availability on aerobic NO detoxification in *E. coli*. Font shading reflects qualitative, relative abundance (bold dark: highest). Glucose (Glc) is the carbon source depicted, – indicates a lack of Glc. Initial state corresponds to normal aerobic physiology of *E. coli*. When stressed with NO with sufficient Glc (replete), NADH remains high, Hmp is synthesized, and NO is detoxified. When NO is encountered in the presence of limited quantities of Glc, Hmp is synthesized and NADH levels support NO detoxification until Glc is exhausted. At that point, Hmp drains NADH and NO detoxification ceases. When stressed with NO in the absence Glc (starved), Hmp cannot be synthesized and NO cannot be detoxified, which has a protective effect on NADH levels, rendering them higher than exhausted conditions, although lower than replete conditions.

manner that does not depend on respiration, which is inhibited by NO [34]. The data presented here adds to this knowledgebase by providing deeper understanding of how aerobic NO detoxification in *E. coli* fails due to carbon source availability, which we anticipate will be useful for future investigations of NO stress in non-ideal, physiologically-relevant nutritional environments.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.05.082.

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