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Original Contribution

Exploring the redox balance inside gram-negative bacteria with redox-sensitive GFP

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ABSTRACT

Aerobic bacteria are continuously fighting potential oxidative stress due to endogenous and exogenous reactive oxygen species (ROS). To achieve this goal, bacteria possess a wide array of defenses and stress responses including detoxifying enzymes like catalases and peroxidases; however until now, the dynamics of the intra-bacterial redox balance remained poorly understood. Herein, we used redox-sensitive GFP (roGFP2) inside a variety of gram-negative bacteria to study real-time redox dynamics immediately after a challenge with hydrogen peroxide. Using this biosensor, we determined the individual contributions of catalases and peroxidases and found that each enzyme contributes more to rapid detoxification or to prolonged catalytic activity. We also found that the total catalytic power is affected by environmental conditions. Additionally, using a *Salmonella* strain that is devoid of detoxifying enzymes, we examined endogenous ROS production. By measuring endogenous ROS production, we assessed the role of oxidative stress in toxicity of heavy metals and antibiotics. We found that exposure to nickel induced significant oxidative stress whereas cobalt (which was previously implicated to induce oxidative stress) did not induce ROS formation. Since a turbulent debate evolves around oxidative stress as a general killing mechanism by antibiotics (aminoglycosides, fluoroquinolones and β-lactams), we measured oxidative stress in bacteria that were challenged with these antibiotics. Our results revealed that antibiotics do not induce ROS formation in bacteria thereby disputing a role for oxidative stress as a general killing mechanism. Together, our results expose how the intra-bacterial redox balance in individual microorganisms is affected by environmental conditions and encounters with stress-inducing compounds. These findings demonstrate the significant potential of roGFP2 as a redox biosensor in gram-negative bacteria to investigate redox dynamics under a variety of circumstances.

1. Introduction

Reactive oxygen species (ROS) plague all microorganisms that live in oxygenated environments. Either through exposure to exogenous ROS that are present in the environment or endogenous ROS, bacteria experience various degrees of oxidative stress. The term “reactive oxygen species” includes a wide variety of different oxygen radicals that all have specific reactivity to certain biomolecules and therefore can have profoundly different effects on bacteria. Parameters that are important for understanding interactions between specific ROS and bacteria, include the spatial localization of ROS and their respective concentration. For example, hydrogen peroxide is known to exhibit bacteriostatic actions at lower concentrations, whereas at higher concentrations, hydrogen peroxide directly interacts with DNA and proteins eventually leading to bacterial cell death [1,2].

Bacteria have evolved numerous defense mechanisms against oxidative stress. An important first line of defense is the production of detoxifying enzymes including catalases, peroxidases and superoxide dismutases. Together, these enzymes convert superoxide and hydrogen peroxide into water and oxygen. After damage to intra-bacterial components, specific enzymes are produced to repair biomolecules and restore a healthy environment. Another defense strategy is to use glutathione as a buffering molecule to maintain a stable intra-bacterial redox balance. Many gram-negative bacteria use glutathione as their buffering low-molecular weight thiol for maintaining a stable redox environment [3,4].

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Bacterial defenses against oxidative stress are regulated by specific transcriptional regulators e.g. OxyR or SoxR, that can "sense" oxidative stress [5]. Oxidation of these transcription factors leads to a conformational change in the protein and a corresponding change in DNA-binding properties. After oxidative stress has occurred, specific genes are switched "on" while others are switched "off". Bacterial catalases and peroxidases are examples of genes that are regulated by these transcription factors.

Despite many years of research, measuring oxidative stress inside bacteria remains challenging and a variety of experimental approaches have generated results that are often ambiguous or contradictory [6,7]. A clear example of this is illustrated by the recent debate over oxidative stress as a general antimicrobial mechanism caused by antibiotics. These discussions are fueled by conflicting data over the potential involvement of ROS in bacterial killing by antibiotics [8–13]. New analytical tools for the measurement of oxidative stress in bacteria are desperately needed to resolve some of these uncertainties. Traditional methods for analyzing oxidative stress often rely on the conversion of a fluorescent dye after interaction with specific ROS. Although these measurements can generate useful results, dyes can sometimes be affected by unrelated chemical events and their output relies on the concentration of dye inside the cell which can vary for different experimental conditions [6]. Moreover, since the conversion of fluorescent dyes is mostly irreversible, these methods provide no useful information about dynamics or detoxification of ROS inside bacteria. More recent approaches that rely on induction of oxidation-sensitive promoters that drive GFP or RFP expression have shown promise in the study of bacterial ROS encounters that happen over longer periods of time [14,15]. However, since ROS concentrations are known to fluctuate rapidly, there is an urgent and unmet need for the development of methods that can measure rapid redox fluctuations in the intra-bacterial redox environment in real time. Recently, we described the use of redox-sensitive GFP (roGFP2) inside *Salmonella enterica* Typhimurium to measure real-time changes to the intra-bacterial redox balance [16]. The roGFP2-biosensor was engineered to contain specific cysteines that form a disulfide bond upon oxidation [17]. Formation of the disulfide bond leads to a slight shift in protein conformation and the resulting oxidized and reduced isoforms of the protein can be distinguished by differential fluorescence after excitation at 405 nm and 480 nm, respectively. The ratio of fluorescent signal after excitation at 405 and 480 nm can be used to calculate the redox potential and is a continuous measure reporting the intra-bacterial redox balance [18]. Because roGFP2 reports the redox balance by ratio-metric analysis, this system excludes variations due to differences in roGFP2 concentrations. Despite extensive use of roGFP in eukaryotic cells [3,17,19–23], this biosensor has only recently been used in bacterial systems [16,24]. Although other groups have created fusions between roGFP2 and catalyzing enzymes to speed up the response time and increase specificity [3,25], we found that the response of unaltered roGFP2 in bacteria was immediate [16,18]. In order to ensure sensitivity to different sources of redox stress, we used unaltered roGFP2 for our analyzes.

First, we tested roGFP2 in a variety of different gram-negative bacteria to explore differences in stress responses between bacterial species. By real-time monitoring of the intra-bacterial redox balance, we analyzed the length of time required to detoxify various amounts of hydrogen peroxide (H_2O_2). We were able to use this information to calculate the catalytic activity of detoxifying enzymes in several bacterial species and measure the catalytic activity in different environmental conditions. We also studied the contributions of individual detoxifying enzymes in *S.* Typhimurium. Finally, we investigated the role of metal ions in bacterial detoxification and tested endogenous ROS generation after exposure to metal ions and antibiotics. These results provide major insights into the complex nature of redox dynamics that occurs in gram-negative bacteria.

In this manuscript, we mostly focus on disruption of the intra-bacterial redox balance resulting from exposure to exogenous hydrogen peroxide or from endogenous ROS produced within bacteria. If the exact oxygen radicals in an experimental set up were known to us, we refer to these radicals by their specific names. However, when the production of endogenous ROS was examined, the cocktail of radicals was more undefined and we chose to refer to these radicals with the more broad terminology of ROS.

2. Material and methods

2.1. Cloning roGFP2

The roGFP2 gene used in this study, originated from the pRSETB vector [17]. This gene was cloned into the high copy pfpv25 vector for constitutive expression of roGFP2 in gram-negative bacteria. The pfpv25 vector carried the RpsM promoter from *S.* Typhimurium [26]. The pfpv25-roGFP2 vector was transformed into target strains and maintained by addition of 100 μg/ml carbenicillin [16].

2.2. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Most gene deletions were made in the *S.* Typhimurium (12023) background. Deletions and concomitant insertion of an antibiotic resistance cassette were done using λ-red-mediated recombination as was described previously [27]. In short, mutations were moved by P22 transductions. In order to avoid outgrowth of suppressed strains, *katE*, *katG*, *katN*, *ahpCF* and *tsaA*, mutations were selected anaerobically on LB agar supplemented with bovine liver catalase (2000 U/plate). In some cases, antibiotic resistance cassettes were removed by using the temperature-sensitive plasmid

Table 1
Bacterial strains used in this study.

Strain	Relevant characteristics	Source or reference
***Salmonella enterica* Typhimurium (SL1344)**		[28]
oxyR	*oxyR*::kan	[29]
Salmonella enterica		
kat-	Δ*katE*, Δ*katG*, *katN*::kan	[27]
ahp-	ahpCF::kan tsaA::cat	[27]
hpxf	Δ*katE*, Δ*katG*, Δ*katN*, Δ*ahpCF*, Δ*tsaA*	[27]
katE	Δ*katE*	This study
katG	Δ*katG*	This study
katN	Δ*katN*	This study
ahpCF	Δ*ahpCF*	This study
tsaA	Δ*tsaA*	This study
***Escherichia coli* (DH10B)**		[30]
***Escherichia coli* (BL21)**		[31]
***Salmonella enterica* Typhi (TY2)**		[32]
***Salmonella enterica* Typhi (ISP1820)**		[33]
Enteropathogenic *E. coli* (EPEC) O127:H6 (E2348/69)		[34]
Enterohaemorrhagic *E. coli* (EHEC) O157:H7 (86-24)		[35]
***Citrobacter rodentium* (DBS100)**		[36]
***Yersinia pseudotuberculosis* (YPIII)**		[37]