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Modulation of bacterial virulence and fitness by host glutathione

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Glutathione is a low molecular weight thiol that is important for maintaining intracellular redox homeostasis. Some bacteria are able to import exogenous glutathione as a nutritional source and to counter oxidative stress. In cytosolic pathogens *Burkholderia pseudomallei* and *Listeria monocytogenes*, host glutathione regulates bacterial virulence. In *B. pseudomallei*, glutathione activates the membrane-bound histidine kinase sensor VirA that leads to activation of the Type VI Secretion System. In *L. monocytogenes*, host glutathione leads to the binding of bacterial glutathione to the master virulence regulator PrfA as an allosteric activator. Glutathione can also modulate virulence factors to control their activity by S-glutathionylation. Thus, host glutathione acts as a spacio-temporal cue for some pathogens to switch on their virulence programs at the right time and place.

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Introduction

Glutathione, also known as L-gamma-glutamyl-L-cysteinylglycine, is a low molecular weight thiol that plays a pivotal role in numerous biological processes [1]. Intracellular glutathione is predominantly (>98%) in the thiol-reduced form (GSH) with the remaining amounts undergoing thiol oxidation to form glutathione disulfide (GSSG) and mixed disulfides with target proteins [2]. It is the most abundant intracellular thiol in eukaryotic and many prokaryotic cells, with concentrations in the millimolar range [1,3]. It is synthesised by Gram-negative bacteria [4] but only a few Gram-positive bacteria, such as *Listeria monocytogenes*, make GSH. The sulfhydryl group (thiol) of the cysteine is involved in oxidation and reduction, providing the means for removal of reactive species and xenobiotic compounds [5]. Glutathione is important in the control of intracellular redox homeostasis, cell signalling, and salvage of the essential amino acid cysteine [6,7].

Because of its central role as the dominant redox couple, deletion of genes involved in glutathione synthesis in bacteria generally affects the fitness of the bacteria in oxidative stress environments, including *in vivo* infections. This review discusses the role of exogenous rather than endogenous glutathione in modifying bacterial physiology and virulence.

Glutathione modulation of virulence regulators in *Burkholderia pseudomallei* and *L. monocytogenes*

Recent evidence shows that extracellular glutathione could directly modulate bacterial virulence. The two examples are both facultative intracellular pathogens, where host glutathione acts as a signal for bacteria to turn on their virulence programs when they are inside host cells.

B. pseudomallei is the causative agent for melioidosis, and it can infect a wide variety of mammalian cells [9,10]. Upon entry by yet ill-defined mechanisms, it escapes from the oxidizing environment of the phagosome via its Type 3 Secretion System 3 (T3SS3) into the host cytosol, where it multiplies to high numbers [11]. The bacterium then uses the abundant GSH from the host cytosol to upregulate expression of Type 6 Secretion System 5 (T6SS5). T6SS5 is a critical virulence cluster encoding a secretion system that is essential for bacterial pathogenesis inside mammalian hosts [12••]. T6SS5 allows the bacterium to fuse host cells to form multinucleated giant cells (MNGCs), resulting in intercellular spread [13]. Mice infected with T6SS5 mutants cleared the infection, demonstrating that T6SS5 is a critical virulence factor in mammalian infection. As *B. pseudomallei* exits the phagosome into the host cytosol, it experiences a rapid change from an oxidizing to reducing environment. *B. pseudomallei* senses entry into the reducing environment through the action of GSH on its two-component sensor/regulator system VirA and VirG. GSH in the cytosol is transported into the bacterial periplasm through unknown mechanisms to reduce VirA. VirA is a histidine kinase sensor protein present on the inner membrane, its reduction on a cysteine residue predicted to be situated in the periplasm drives a switch from a dimeric state into the monomeric state, thereby activating T6SS through the response regulator VirG [12••]. Thus,

B. pseudomallei uses host GSH as a 'Global Positioning System' signal to indicate that it has successfully escaped from the hostile oxidizing environment of the phagocytic vacuole and entered the cytosol where it thrives.

L. monocytogenes, is a Gram-positive foodborne pathogen. It uses GSH to turn on its virulence program through the master virulence regulator PrfA in a more complicated, two-step strategy [15••]. Extracellular GSH and other reducing agents are able to trigger the intracellular production of bacterial GSH through unknown mechanisms [16••]. The increasing concentrations of endogenous GSH bind to PrfA and function as an allosteric activator [15••]. Modification of PrfA turns on a virulence program involving 10 core genes directly affected by PrfA and another 145 genes that are indirectly affected [17]. The crystal structures of the PrfA-GSH and PrfA-GSH-DNA complex show that GSH binding to PrfA induces a fold in the winged helix-turn-helix (HTH) motif of PrfA at the C terminal end that is responsible for binding to the DNA operator region [18•]. This induces bending of the DNA and increases the ratio of active versus inactive PrfA. Even though the binding affinity of PrfA to GSH is quite low (4 mM [15••]), the high physiological concentrations of GSH in the cytosol of both prokaryotes and eukaryotes (0.1 mM–10 mM) would still favour such an interaction.

The similarities and differences between the effect of GSH on *Burkholderia* and *Listeria* are summarised in Figure 1. These two pathogens are very different genetically, but through convergent pathways have evolved to exploit host GSH in the cytosol as a spacio-temporal cue for the same purpose of activating their virulence programs.

Because glutathione functions as a lifestyle switch, it is possible that it can also modify expression of metabolic genes necessary for cytosolic survival, in addition to modifying bacterial virulence. In an RNAseq experiment with 2 mM of GSH added to *B. pseudomallei*, we found upregulation of genes involved in the vitamin B12 biosynthetic pathway, and pyochelin, an iron siderophore (Table 1). *B. pseudomallei* possesses the biosynthetic pathway for vitamin B12 synthesis. In *Mycobacterium tuberculosis*, vitamin B12 was necessary for methionine synthesis and propionate utilization [19]. One could speculate that vitamin B12 is important for metabolism of *Burkholderia* in the cytosol. Although we do not yet understand how GSH contributes to the regulation of these genes and what their roles are in cytosolic survival, we postulate that they would be good candidates for maintaining an intracellular lifestyle.

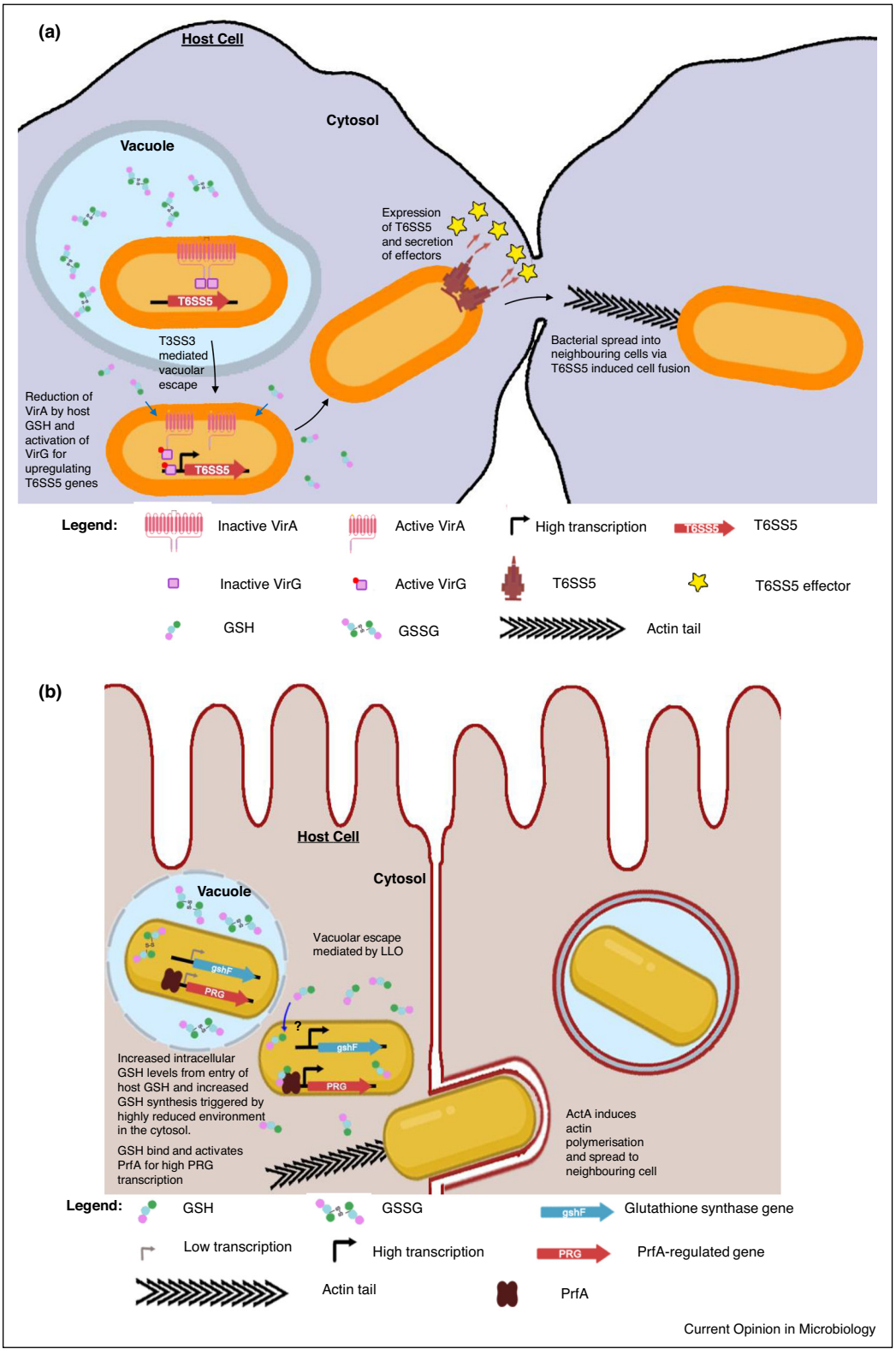
Glutathione modulation of transcriptional activity in *Escherichia coli*

Do extracellular bacteria similarly use GSH as a signal to regulate lifestyle changes? *E. coli* has an oligopeptide transporter encoded by the *yliABCD* operon that imports glutathione [20]. A recent study reported a transcriptomic profiling of *E. coli* MG1655 laboratory strain to exogenously added GSH at a concentration of 10 mM. In comparing bacteria with or without GSH supplementation, the most prominent group of genes identified and verified by qRT-PCR were associated with the acid shock response [21]. As an aside, the GSH stock solution used in that study was dissolved in water and then added to LB. This is not ideal, the pH changes drastically upon addition of GSH or GSSG in LB (Table 2). At 10 mM, the pH of LB dropped to 4.62, which could explain the strong acid shock response reported in the study. It is thus critical that studies using high concentrations of GSH consider the effect of pH, and neutralize the acidity by the addition of a base such as sodium hydroxide. Therefore, the transcriptional regulatory effects exerted by exogenous GSH on *E. coli* are still unclear. The environment in the intestinal tract is anaerobic and highly reducing. The GSH concentrations in the intestinal epithelium have been reported to be between 2–10 mM [22], providing a rationale for examining the effect of GSH on the physiology of *E. coli* and perhaps even its virulence in the gut.

The effect of S-glutathionylation on bacterial virulence

In addition to its role as a lifestyle switch via activation of bacterial transcriptional regulators, glutathione also alters bacterial virulence through S-glutathionylation. Protein S-glutathionylation is the specific post-translational modification (PTM) of cysteine residues by the addition of glutathione [2]. This process occurs in response to oxidative and nitrosative stress, and protects sensitive protein thiols from irreversible oxidation. It may also serve as a storage form of glutathione. S-glutathionylation has been well described to regulate signalling pathways in eukaryotes, but is less understood in bacteria. Recently, a comprehensive top down proteomics approach in *Salmonella* identified different forms of protein S-thiolation that functioned as a switch between infection (mimicked by a low-phosphate, low-magnesium, low-pH minimal medium) and basal (LB) conditions [23•]. Under infection-like conditions, *Salmonella* preferentially used S-cysteinylation as a mechanism for thiol protection and/or environmental sensing, but switched to S-glutathionylation under basal conditions. The authors hypothesized that cysteine was preferentially used instead of glutathione under infection-like conditions because glutathione requires additional synthesis compared to cysteine. Thus, glutathione would not be energetically favourable under stress conditions. This is certainly plausible, although in an actual animal infection, *Salmonella* is likely exposed to host glutathione. If *Salmonella* has the ability to transport host glutathione efficiently, the distinction between the two forms of S-thionylation may not be so clear. Furthermore, LB contains high concentrations of glutathione, particularly in the yeast extract, and it is unclear whether

Figure 1



GSH induction of virulence genes during *B. pseudomallei* and *L. monocytogenes* infection.
(a) *B. pseudomallei* invade host cells by poorly defined mechanisms and are internalized into single-membrane endosomes. Escape from the vacuole into the cytosol via T3SS3 subjects the bacterium to a strongly reducing environment containing high concentrations of host GSH. Host GSH triggers the reduction of VirA from a dimeric form to a monomeric form that drives the activation of its cognate response regulator, VirG, and

Table 1

***B. pseudomallei* genes upregulated at least 10 fold upon GSH treatment (excluding T6SS5 genes)**

Gene name	Fold change	Gene function	Description
cobK	38.8	CobK	Precorrin-6X reductase activity, converts precorrin-6 into dihydro-precorrin 6 in the cobalamin (vitamin B12) biosynthetic pathway
BPSL1112	23.7	Putative lipoprotein	
BPSL1051	21.1	Pseudogene	
BPSL0774	18.6	Two component system sensor kinase	
pchB	16.1	PchB	Isochorismate-pyruvate lyase activity, produces salicylate in the pyochelin (siderophore for iron acquisition) biosynthetic pathway

Table 2

Effect of GSH and GSSG on pH of LB

Concentration (mM)	GSH	GSSG
0	6.89	6.89
0.2	6.86	6.81
2	6.41	6.01
5	5.47	4.84
10	4.62	4.15
20	3.92	3.63

this medium represents a true basal condition for *Salmonella* outside of the laboratory. Nevertheless, the study points to future possibilities of performing comprehensive PTM analyses of bacterial proteins in infected host tissues that will reveal the S-thionylation status of bacterial proteins during infection.

In *L. monocytogenes*, there is evidence that S-glutathionylation affects bacterial virulence through its action on Listeriolysin O (LLO) [24••]. The primary role of LLO is to mediate escape of the bacteria from the phagosome to the cytosol, and reduction of LLO is necessary for its activation. Reversible S-glutathionylation on the lone cysteine residue in LLO inactivates the protein. It was postulated that gamma interferon induces a lysosomal thiol reductase that is located in vacuoles of macrophages and this enzyme could reduce the S-glutathionylated LLO, resulting in its activation and subsequent bacterial escape from the vacuole. Alternatively, S-glutathionylation might protect LLO from irreversible oxidation in the phagosome.

Another recent example of S-glutathionylation is on LcrV, a T3SS protein in *Yersinia pestis* [25••]. This is an interesting example, because LcrV is secreted by the bacteria to form the cap of the T3SS. LcrV is modified by host-derived glutathione following its export to the tip of the type III secretion machine. S-glutathionylation of LcrV moderates and impedes the rate of *Y. pestis* type III effector injection into immune cells, and enhances *Y. pestis* virulence in rodent models of bubonic plague. It also promotes the association of LcrV with host RPS3, a multifunctional cytosolic protein that regulates DNA repair, apoptosis and innate immune responses. Association of S-glutathionylated LcrV with RPS3 leads to suppression of apoptosis of *Y. pestis*-infected macrophages [25••]. The authors suggested that the abundance of glutathione and the strong oxidizing environment in mammalian blood promoted LcrV glutathionylation, which led to necroptotic cell death and IL-1β and IL-18 secretion. The enhanced inflammation that ensued would lead to more killing of immune cells and enhanced dissemination of bacteria in host tissues, increasing pathogenesis.

Glutathione import promotes survival in the host

In *Francisella tularensis*, a screen of Himar1 transposon mutants for defects in intracellular growth resulted in the identification of a mutant in gamma glutamyl transpeptidase (GGT) [26]. The authors discovered that host glutathione was broken down by GGT into cysteine. *Francisella* is a cytosolic pathogen, and the abundance of cytosolic glutathione provides a convenient source of cysteine to enable intracellular growth and replication. GGT has also been shown to enable *E. coli* to use glutathione as a source of cysteine [27]. The obligate human pathogen *Hemophilus influenza* imports glutathione by the dipeptide ABC transporter DppBCDF primed

(Figure 1 Legend Continued) the expression of T6SS5 genes. T6SS5 assembly and secretion of effectors induce cell-to-cell fusion and facilitates the spread of *B. pseudomallei* to its neighbouring cells.
(b) After the invasion of *L. monocytogenes* into host cells, secreted pore forming toxin listeriolysin O (LLO), ruptures the vacuole and enables bacterial escape into the cytosol. The strongly reducing environment of the cytosol containing high concentrations of host GSH triggers *gshF* gene transcription via an unknown mechanism and increases bacterial GSH synthesis. GSH that is synthesised by the bacteria or via the transportation from the host cytosol binds transcriptional regulator PrfA allosterically and activates the transcription of PrfA-regulated genes (PRGs). An example of a PRG is ActA. Expression of ActA enables the polymerization of host actin for propelling the bacterium through membrane protrusions into neighbouring cells.

with the periplasmic-binding protein GbpA, to counter high oxidative stress encountered during colonization of the human upper respiratory tract [28].

The Gram-positive pathogen *Streptococcus pneumoniae*, does not synthesize glutathione, but it possesses GshT, an ABC transporter substrate binding protein that imports exogenous glutathione [29]. A *gshT* mutant was hypersensitive to oxidative stress and challenge with divalent metal ions such as copper, zinc and cadmium. It was also attenuated in a mouse model of pneumococcal colonization and invasion. The importance of glutathione in protection of other lactic acid bacteria from oxidative stress is evident in *Streptococcus mutans*, where glutathione protects from challenge with the thiol-specific oxidant diamide [30]. Glutathione is also a source of sulfur amino acids [31]. In *Lactococcus lactis*, glutathione confers resistance to H_2O_2 [32].

Conclusions

Bacteria import exogenous glutathione mainly as a source of cysteine for growth and as protection against oxidative stress or metal toxicity. These include bacteria that make their own glutathione as well as those that do not. This protective mechanism supplements endogenous defenses in stressful environments such as during host infection, and could be particularly useful for oxidative reactions occurring near the bacterial surface. Apart from the traditional understanding of the role of glutathione, there is now evidence that glutathione participates in S-glutathionylation to modify bacterial fitness and virulence. This area deserves further investigation to understand whether post-translational modifications regulate the activity of additional virulence factors. This is particularly interesting for toxins and secretion systems, as these typically contact host membranes. Thus, they could be exposed to the membrane and cytosol interface, where a redox difference exists. Perhaps the most exciting realization is that glutathione modifies key virulence transcriptional activators to regulate a lifestyle switch in intracellular bacteria. This is an ingenious adaptation by cytosolic pathogens to co-opt an abundant and essential molecule of the host as a signal to turn on their virulence programs. One unresolved issue is how exogenous glutathione is transported into *Burkholderia* and *Listeria*. Another exciting question is whether there are more bacteria with a similar strategy of using exogenous glutathione to control a lifestyle switch, whether it is from transit into the host cytosol or upon entry into a reducing host environment such as the intestine. The answers await future discoveries.

Conflict of interest statement

Nothing declared.

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