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Glutathione activates virulence gene expression of an intracellular pathogen

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Abstract

Intracellular pathogens are responsible for much of the world-wide morbidity and mortality due to infectious diseases. To colonize their hosts successfully, pathogens must sense their environment and regulate virulence gene expression appropriately. Accordingly, on entry into mammalian cells, the facultative intracellular bacterial pathogen *Listeria monocytogenes* remodels its transcriptional program by activating the master virulence regulator PrfA. Here we show that bacterial and host-derived glutathione are required to activate PrfA. In this study a genetic selection led to the identification of a bacterial mutant in glutathione synthase that exhibited reduced virulence gene expression and was attenuated 150-fold in mice. Genome sequencing of suppressor mutants that arose spontaneously *in vivo* revealed a single nucleotide change in *prfA* that locks the protein in the active conformation (PrfA*) and completely bypassed the requirement for glutathione during infection. Biochemical and genetic studies support a model in which glutathione-dependent PrfA activation is mediated by allosteric binding of glutathione to PrfA. Whereas glutathione and other low-molecular-weight thiols have important roles in redox homeostasis in all forms of life, here we demonstrate that glutathione represents a critical signalling molecule that activates the virulence of an intracellular pathogen.

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Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Listeria monocytogenes is a Gram-positive pathogen of animals and humans that cycles between a saprophytic lifestyle and an intracellular pathogen that escapes from a vacuole and grows in the cytosol of host cells[1]. The intracellular lifecycle of *L. monocytogenes* has been well characterized and is entirely dependent on the transcription factor PrfA (refs 2, 3). PrfA directly regulates the transcription of nine virulence factors and is therefore referred to as the master virulence regulator in *L. monocytogenes*[4]. In keeping with its central role in pathogenesis, *L. monocytogenes* strains lacking *prfA* are completely avirulent[1,3]. PrfA is a member of the cAMP receptor protein (Crp) family of transcription factors, which are characterized by their allosteric regulation via small-molecule activators. In *L. monocytogenes*, PrfA is exclusively activated in the cytosol of host cells, leading to the assumption that the activating cofactor for PrfA is specific to this compartment. However, even after decades of study, the biochemical mechanism by which PrfA detects the intracellular environment is not well understood. The goal of this study was to identify how *L. monocytogenes* recognizes and responds to its intracellular niche of the mammalian cell cytosol.

Genetic selection in macrophages

We devised a genetic selection to isolate bacterial mutants unable to activate virulence genes during intracellular growth. Our strategy took advantage of a *L. monocytogenes* vaccine strain designed to die *in vivo* (P.L. *et al*., manuscript in preparation). Specifically, *loxP* sites were inserted into the *L. monocytogenes* chromosome flanking the origin of replication (*ori*, Fig. 1a). Into this background a codon-optimized *cre* recombinase gene was inserted under the control of the *actA* promoter, which is the most exquisitely regulated PrfA-dependent virulence gene in *L. monocytogenes* and is specifically activated in the host cytosol[2,3,5,6]. The resulting strain grew like wild type *in vitro* (Fig. 1b) where *actA* expression is very low[4,5]. However, on cytosolic access, Cre-mediated recombination of the *loxP* sites resulted in excision of the *ori*, preventing bacterial replication (Fig. 1c). A transposon library was then generated in this ‘suicide’ strain background. Bone-marrow-derived macrophages (BMDMs) were infected with the library of transposon mutants and the surviving bacteria were recovered.

We identified more than 16 independent insertions in a *L. monocytogenes* gene, previously identified as encoding a bifunctional glutathione synthase (*gshF*)[7], that rescued the death of the ‘suicide’ strain *in vivo* (Fig. 1c, d). Glutathione is a tripeptide low-molecular-weight (LMW) thiol present in all eukaryotes that contain mitochondria and nearly all Gram-negative bacteria[8]. *L. monocytogenes* is one of the few Gram-positive bacteria that synthesize glutathione, whereas many utilize alternative LMW thiols such as bacillithiol and mycothiol[9,10]. Glutathione was not required for Cre/*lox* recombination when *cre* was expressed from a constitutive promoter (data not shown), leading to the hypothesis that glutathione was required specifically for activation of the *actA* promoter.

Glutathione is required for virulence

To determine the role of *gshF* in *L. monocytogenes*, an in-frame deletion strain was generated by allelic exchange (Δ*gshF*). Consistent with published work[7], the *gshF*-deficient

strain was moderately more sensitive to oxidative stress *in vitro* (Extended Data Fig. 1). However, Δ*gshF* did not suffer a general loss of fitness, as it exhibited no measurable growth defect *in vitro* (Fig. 2a) or in BMDMs (Fig. 2b). As expected based on the criteria of the genetic selection, the Δ*gshF* mutant expressed lower levels of ActA in cells (Fig. 2c), formed very small plaques in tissue culture assays that measure cell-to-cell spread (Fig. 2d), and was greater than 2-logs less virulent in mice (Fig. 2e). Complementation of Δ*gshF* with its native promoter (Δ*gshF* + *gshF*) restored wild-type ActA abundance, wild-type plaque size, and virulence (Fig. 2c–e). Since all mammalian cells have high intracellular levels of glutathione[11], we assessed the potential contribution of host glutathione using buthionine sulfoximine (BSO). BSO depletes total cellular glutathione levels >98%[12], but had no effect on bacterial growth during infection (Extended Data Fig. 2). Whereas wild-type *L. monocytogenes* was unaffected, the Δ*gshF* mutant failed to synthesize detectable ActA in the BSO-treated cells (Fig. 2f). These results demonstrated that the remaining ActA expression in the Δ*gshF* mutant was due to imported host glutathione and also established that the phenotypes observed for Δ*gshF* were due to a lack of glutathione and not absence of the GshF protein.

Isolation of suppressor mutations *in vivo*

To elucidate the role of glutathione during infection we sought to isolate suppressor mutations. The selective pressure of the host environment was used to select for compensatory mutations in the Δ*gshF* background that restored virulence to identify functionally interacting genes and/or pathways. Since previous work identified *gshF::*Tn mutants as hypo-haemolytic[13], we screened for hyper-haemolytic colonies from the liver homogenates of infected animals on blood agar plates. Two hyper-haemolytic colonies were isolated and genome sequencing identified a single nucleotide polymorphism (SNP) common to both strains and absent from the Δ*gshF* parental strain. The SNP encoded a PrfA G145S mutation, which is the most commonly found spontaneous PrfA* allele[14], so called because of its structural similarity to well-characterized Crp* mutants that are constitutively active in the absence of cofactor[15]. The PrfA G145S allele rescued ActA expression and virulence of Δ*gshF*, confirming the function of this mutation identified by our *in vivo* suppressor analysis (Fig. 3a–c). This was not specific to *actA*, as transcript levels of two other PrfA-dependent genes were also restored by the PrfA G145S mutation (Extended Data Fig. 3). Furthermore, two other previously identified PrfA* alleles[16] also rescued the plaque defect of Δ*gshF* (Fig. 3d), indicating that constitutively activating PrfA completely bypassed the requirement for glutathione during infection. Importantly, these data highlighted that Δ*gshF* was not attenuated during infection due to a general loss of fitness, but rather, due to a dysregulation of virulence genes.

PrfA binds glutathione allosterically

In addition to its role in maintaining redox homeostasis, glutathione can be covalently bonded to protein thiols as a post-translational modification, a process referred to as *S*-glutathionylation[17]. To determine whether glutathionylation of PrfA is required for its activation, we engineered a PrfA protein in which all four cysteine residues were mutated to alanine (referred to as PrfA(C38A/C144A/C205A/C229A or PrfA $C/A)_4$). Recombinant

PrfA$(C/A)_4$ bound DNA with an affinity similar to wild-type PrfA *in vitro* (Table 1 and Extended Data Fig. 4), establishing that these mutations do not disturb the overall structural integrity of the protein. However, the cysteine residues were found to contribute to DNA binding, as demonstrated by the 25-fold lower affinity of oxidized wild-type PrfA as compared to reduced PrfA (Table 1). Although PrfA$(C/A)_4$ bound DNA *in vitro*, it was less abundant than wild type when expressed from the native locus on the chromosome of *L. monocytogenes* (Extended Data Fig. 5). Since PrfA is auto-regulated[18], these data suggested that PrfA$(C/A)_4$ was less active *in vivo*. Indeed, the PrfA$(C/A)_4$ strain synthesized less ActA than the wild-type strain during BMDM infection (Fig. 4a) and was 30-fold less virulent in mice (Fig. 4b). Together, these results suggested that the cysteine residues of PrfA were dispensable for DNA binding *in vitro*—as the mutant lacking all cysteine residues (PrfA$(C/A)_4$) bound DNA with an affinity similar that of the wild type (Table 1)—but were required for activity *in vivo*. If glutathionylation of PrfA was important for its activity, then deleting *gshF* in the PrfA$(C/A)_4$ background would have no effect. Remarkably, combining the PrfA$(C/A)_4$ and *gshF*::Tn mutations resulted in a strain that was defective for intracellular growth (Extended Data Fig. 6) and completely avirulent in mice (>4-log attenuation, Fig. 4b).

Although we cannot definitively exclude a role for glutathionylation of PrfA *in vivo*, covalent modification of the protein thiols was not sufficient for PrfA activation, as the PrfA$(C/A)_4$ mutant was only modestly attenuated during infection (Fig. 4b). Therefore, we hypothesized that glutathione may be allosterically binding PrfA, analogous to the interaction of cAMP binding to Crp[19]. The binding affinity of glutathione for PrfA was measured via bio-layer interferometry. A direct and specific interaction with reduced glutathione (GSH) was detected for both recombinant wild-type and PrfA$(C/A)_4$ with binding affinities of 4.4 ± 1.2 and 4.7 ± 1.5 mM, respectively, whereas nomeasurable interaction was found between either protein and oxidized glutathione (GSSG, Table 1). Although the affinity for reduced glutathione appears to be relatively low, it is well within biologically relevant concentrations of this LMW thiol, as both prokaryotes and eukaryotes have intracellular concentrations of 0.1–10 mM glutathione[8,11]. This binding affinity would also allow PrfA to be sensitive to varying concentrations of glutathione, rather than beinga simple ON–OFF switch. In support of the hypothesis that glutathione may activate PrfA *in vivo*, *gshF* was transcriptionally upregulated tenfold during infection (Fig. 4c). These data demonstrate that reduced glutathione non-covalently binds PrfA and support the model that glutathione is the activating cofactor for PrfA.

Discussion

The results of this study clearly demonstrate that both bacterial and host-synthesized glutathione contribute to expression of *L. monocytogenes* virulence factors via allosteric binding of the master virulence regulator PrfA. Unlike Crp, PrfA does not require allosteric activation to bind DNA *in vitro* (Table 1). Indeed, the DNA-binding affinity of PrfA was unchanged in the presence of glutathione (data not shown). In this regard, PrfA is similar to the phylogenetically more closely related Crp family member NtcA from cyanobacteria, which also binds DNA in the absence of its cofactor[20,21]. Together, our results suggest a model whereby PrfA activation is a two-step process requiring reduced protein thiols for

initial DNA binding and allosteric binding of glutathione to PrfA for transcriptional activation (see model in Extended Data Fig. 7). Indeed, eliminating both of these steps, as in the PrfA(C/A)$_4$ *gshF*::Tn mutant, results in a strain that is as attenuated as a Δ*prfA* mutant (Fig. 4b).

Glutathione is present in the cytosol of all host cells so it is perhaps not surprising that intracellular pathogens import it, as is the case with *Francisella tularensis*[22]. What is surprising is that *L. monocytogenes* imports glutathione from the host and also synthesizes it. The results of this study suggest that *L. monocytogenes* uses glutathione concentration to regulate its biphasic lifestyle and the switch from saprophyte to pathogen. This may be a reflection of the broad host range of this pathogen and the fact that glutathione is ubiquitous in host cells, making it a reliable signal of the *L. monocytogenes* cytosolic niche. Perhaps other LMW thiols, such as coenzyme A, mycothiol and bacillithiol have similar activating roles in virulence gene expression in other pathogens.

METHODS

Bacterial strains and cell culture

The *L. monocytogenes* strains used were all in the 10403S background (Extended Data Table 1). Bacteria were cultured in brain heart infusion (BHI) or tryptic soy broth (TSB), which contains less than 0.5 μM glutathione[9]. All media were from Becton Dickinson (New Jersey). For broth growth curves, overnight cultures were diluted 1:100 and optical density at 600 nm (OD_{600}) was measured at each time point using a spectrophotometer. *gshF* (*lmo2770*) was deleted by allelic exchange using the temperature-sensitive plasmid pKSV7 (refs 23, 24). The Δ*gshF* complemented strain was generated by inserting a C-terminal 6×His-tagged copy of *gshF* with its native promoter into the integration vector pPL2 (ref. 25).

Murine L2 fibroblasts were passaged in Dulbecco modified Eagle medium with high glucose (DMEM, Gibco/Invitrogen) supplemented with 1% sodium pyruvate, 1% L-glutamine, and 10% fetal bovine serum (FBS, GemCell) at37°C with 5% CO_2. Bone-marrow-derived macrophages (BMDMs) were cultured in DMEM supplemented with 1% sodium pyruvate, 1% L-glutamine, 20% FBS and 10% 3T3-MCSF supernatant at 37°C with 5% CO_2.

Transposon library generation and genetic selection

A transposon library was generated in the ‘suicide’ strain using *himar1 mariner* transposon mutagenesis, as previously described[13]. BMDMs were then infected with this library of transposon mutants. Cells were collected at various time points after infection, lysed, and surviving bacteria were plated on BHI agar. Individual colonies were then isolated and used to infect BMDMs to confirm the phenotype. To identify the transposon insertion site, colony PCR was performed using primers listed in Extended Data Table 1. A large percentage of the transposon insertion sites were found in the *actA* promoter, *cre*, and each *loxP* site. The fact that we identified multiple transposon insertions in each *loxP* site, which are less than 40 nucleotides, indicates that the genetic selection approached saturation.

We next screened these mutants by plaque assay and mutants with a plaque size, <90% of wild type were included. Finally, intracellular growth curves were performed to ensure that the mutants had a defect only in *actA* expression and not in intracellular growth. Greater than 16 independent insertions were identified in *lmo2770* (*gshF*), making it by far the most over-represented hit in the selection.

Intracellular growth curves

BMDMs were harvested as previously described[26] and 3×10^6 cells were plated in 60 mm non-TC-treated Petri dishes. Cells were infected with a multiplicity of infection (MOI) of 0.1 and growth curves were performed as described previously[27].

Quantitative RT–PCR of bacterial transcripts

For transcript analysis in broth, bacteria were grown overnight in TSB and subcultured 1:100 into 25 ml TSB. Bacteria were harvested at an $OD_{600} = 1.0$. For transcript analysis during infection, BMDMs were platedata density of 3×10^7 cellsin150mm TC-treated dishes and infected with an MOI of 10. One hour post-infection the cells were washed and media containing gentamicin (50 μg ml^{-1}) was added. Four hours post-infection the cells were washed and lysed in 0.1% NP-40 containing RNAprotect Bacteria Reagent (Qiagen). Bacteria were harvested by centrifugation.

After harvesting bacteria from either broth or BMDMs they were lysed in phenol: chloroform containing 1% SDS by vortexing with 0.1 mm diameter silica/zirconium beads (BioSpec Products Inc.). Nucleic acids were precipitated from the aqueous fraction overnight at −80°C in ethanol containing 150 mM sodium acetate (pH 5.2). Precipitated nucleic acids were washed with ethanol and treated with TURBO DNase per manufacturer's specifications (Life Technologies Corporation). RNA was again precipitated overnight and then washed in ethanol. RT–PCR was performed with iScript Reverse Transcriptase (Bio-Rad) and quantitative PCR (qPCR) of resulting cDNA was performed with KAPA SYBR Fast (Kapa Biosystems). Primers used for qPCR are listed in Extended Data Table 1.

Plaque assay

Plaque assays in L2 murine fibroblasts were performed as previously described[28]. Briefly, bacterial cultures were grown overnight at 30°C, then washed and diluted 1:10 in sterile PBS. Six-well dishes containing 1.2×10^6 L2 cells per well were infected with *L. monocytogenes* for 1 h, then washed and overlaid with 3 ml of media containing 0.7% agarose and gentamicin (10 μg ml^{-1}) to prevent extracellular growth. After 3 days at 37°C, an overlay containing gentamicin and neutral red dye (Sigma) was added and stained overnight. The plates were then scanned and analysed with ImageJ software[29].

Immunoblots of infected BMDMs

BMDMs were plated in 12-well dishes at a density of 10^6 cells per well and infected with an MOI of 10. One hour postinfection the cells were washed and media containing gentamicin (50 μg ml^{-1}) was added. Where indicated, 2 mM buthionine sulfoximine (Santa Cruz Biotechnology) was added to cells 16 h before infection and included throughout the infection. Four hours post-infection the cells were washed and harvested in LDS buffer

containing 5% BME. The samples were then boiled and separated by 10% SDS–PAGE. A rabbit polyclonal antibody against the N terminus of ActA[30] and a mouse monoclonal antibody against P60 (Adipogen) were each used at a dilution of 1:5,000. P60 is a constitutively expressed bacterial protein[31] used as a loading control.

Virulence experiments

Six-to-eight-week-old female CD-1 mice (The Jackson Laboratory) were infected intravenously with 1×10^5 colony-forming units (CFU). Forty-eight hours post-infection the mice were euthanized and spleens and livers were harvested, homogenized and plated for enumeration of bacterial burdens. Sample size was chosen based on standards within the field[32,33]. No statistical methods were used to predetermine sample size. Samples were not blinded or randomized. All statistical tests allowed for unequal variance between groups, that is, two-tailed heteroscedastic *t*-test. All animal work was done in accordance with university regulations. Protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley (MAUP# R235-0813B).

In vivo suppressor analysis

Female CD-1 mice were infected intravenously with 1×10^7 CFU of Δ*gshF* for 72 h and the livers were harvested, homogenized and inoculated into broth. New mice were then infected with 1×10^6 CFU of the bacteria from the liver homogenates. Seventy-two hours post-infection the livers were harvested, homogenized and plated on blood agar plates. Two hyper-haemolytic colonies were observed and were chosen for further analysis.

Genome sequencing

L. monocytogenes genomic DNA was extracted (MasterPure Kit, Epicentre) and sequenced by Illumina 50SR (library preparation and sequencing performed by UC Berkeley QB3 Genomic Sequencing Laboratory). Sequencing data were aligned to the 10403S reference genome and SNP/InDel/structural variation was determined (CLC Genomics Workbench, CLC bio) for the Δ*gshF* parent strain and the two hyper-haemolytic suppressor mutants.

Protein purification and binding analyses

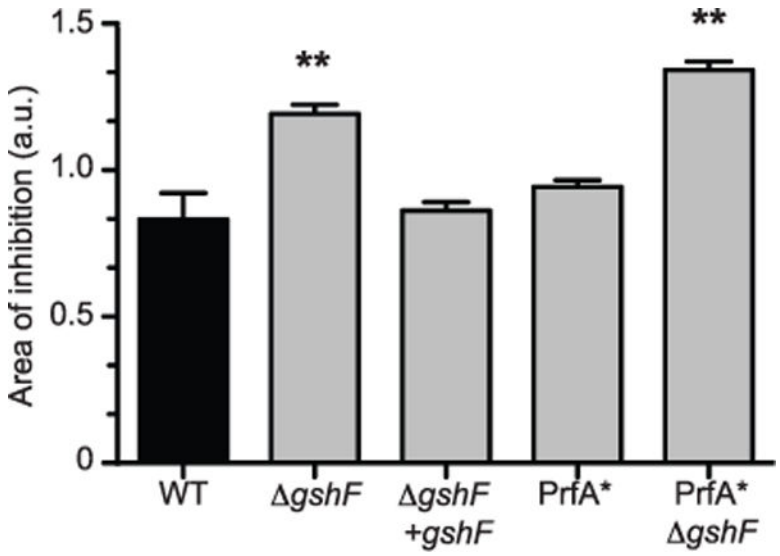
Recombinant PrfA was purified from *Escherichia coli* BL21(DE3) as previously described[34]. Glutathione binding to the wild-type or PrfA$(C/A)_4$ protein was measured by bio-layer interferometry on an Octet RED 384 instrument (Pall ForteBio). The buffer used was PBS, pH7.3 containing 2 mM tris(2-carboxyethyl)phosphine (TCEP) when appropriate. Samples or buffer were dispensed into 384-well microtitre plates at a volume of 100 μl per well. Operating temperature was maintained at 26°C with 1,000 rpm rotary agitation. Ni-NTA biosensor tips (Pall FortèBio) were pre-soaked for 10 min with buffer to establish a baseline before protein immobilization. 6×His-taggedproteins diluted in PBS, pH 7.3 were immobilized onto the biosensors for 8 min at a concentration of 35 μg ml^{-1}. The immobilization level attained was 7–8 nm. Binding association of the glutathione with biosensor tips was monitored for 30 s, and subsequent disassociation in buffer was monitored for 30 s. Glutathione was tested at concentrations of 0.5, 1, 1.5, 2, 3, 4, 5 mM. Reduced glutathione (GSH) was diluted in buffer containing TCEP, while oxidized

glutathione (GSSG) was diluted in PBS only. To control for background, association and dissociation of GSH/GSSG was measured with biosensor tips loaded with buffer only and biosensor tips loaded with protein were tested for binding in buffer with or without TCEP. The apparent affinities of glutathione and PrfA were calculated from equilibrium measurements and, when appropriate, global fits of the k_{on} and k_{off} values, yielding similar values. Data are mean and s.e.m. of experiments from four independent protein preparations.

Fluorescence polarization

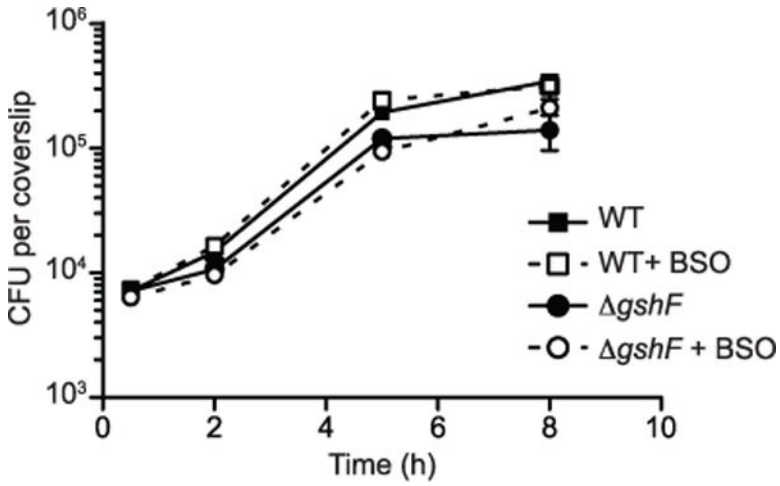
A fluorescence-polarization-based DNA-binding assay was used to determine the affinities of PrfA, PrfA* and the PrfA(C/A)$_4$ mutant for the P*hly* and P*actA* promoters. The sequences of the top strands of P*hly* and P*actA* used in this study are in Extended Data Table 1. The oligodeoxynucleotides were purchased from IDT (Coralville, Iowa) with a fluorescein label covalently attached to the 5′ end. DNA binding was measured in PBS buffer (11.8 mM Na^+/K^+ phosphate, 2.7 mM KCl, 137 mM NaCl) at 25°C using 5 nM fluoresceinated target dsDNA, and 1 μg poly(dI-dC) as a nonspecific DNA competitor. In some experiments 1 mM TCEP was included to maintain a reducing environment. PrfA was titrated into the DNA until saturation as denoted by no further change in the millipolarization (mP = units of polarization × 10^{-3}). The fluoresceinated DNA was excited at 490 nm and its parallel and perpendicular emission intensities measured at 530 nm and converted to units of mP using a Beacon 2000 Variable Temperature Fluorescence Polarization System. Data were plotted and analysed with the following equation: $mP = \{(mP_{bound} - mP_{free})[protein]/K_d + [protein]\} + mP_{free}$, where mP is the millipolarization measured at a given protein concentration, mP_{free} is the initial millipolarization of free fluorescein-labelled DNA, mP_{bound} is the maximum millipolarization of specifically bound DNA, and [protein] is the protein concentration. The generated hyperbolic curves are fit by nonlinear least-squares regression analysis, assuming a bimolecular model such that the K_d values represent the protein concentration at half-maximal ligand binding and plotted by using the graphing program, Kaleidograph. The K_d values are expressed in terms of PrfA dimer binding.

Extended Data



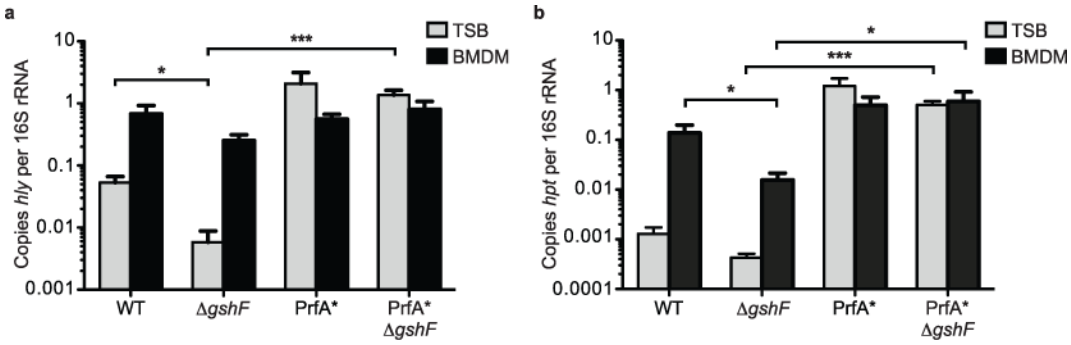
Extended Data Figure 1. *Listeria monocytogenes* Δ*gshF* is sensitive to hydrogen peroxide

Bacteria were grown overnight in TSB and then inoculated into top agar and spread on tryptic soy agar plates. Sterile disks soaked in 10 μl of 15% H_2O_2 (Thermo Fisher Scientific) were placed on the agar and incubated overnight. Plates were then scanned and the area of inhibition was measured (in arbitrary units) using ImageJ software (http://rsbweb.nih.gov/ij). The mean ± s.e.m. of four independent experiments is shown. *P* values were calculated using Student's *t*-test; $^{**}P < 0.01$. a.u., arbitrary units.



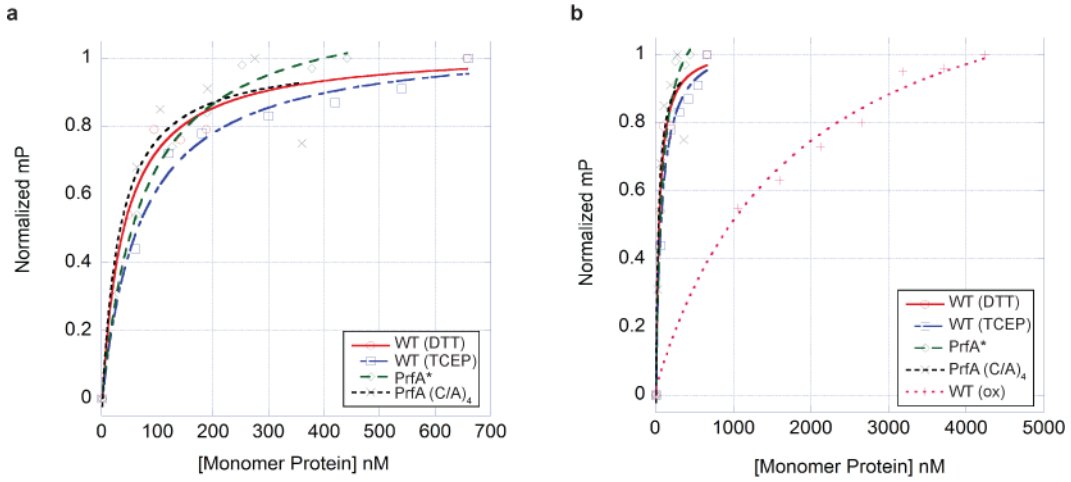
Extended Data Figure 2. BSO does not affect *L. monocytogenes* growth

BMDM growth curve in which cells were untreated or treated with 2 mM BSO for 16 h before infection and throughout the infection. The mean ± s.e.m. of three independent experiments is shown.



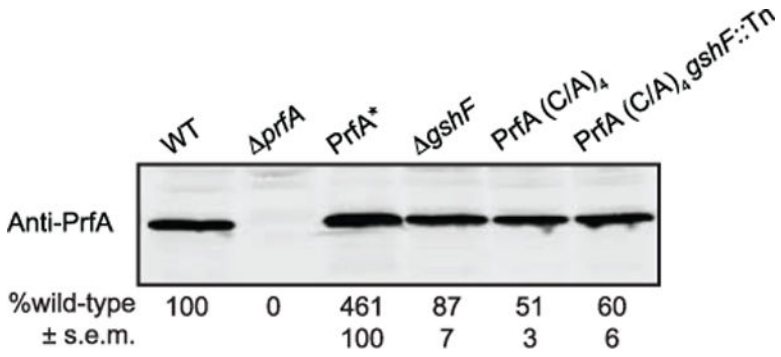
Extended Data Figure 3. The effect of Δ*gshF* is not specific to *actA* regulation

Quantitative RT–PCR of *hly* (**a**) or *hpt* (**b**) transcript levels. Bacteria were harvested from TSB at mid-log (grey bars) or 4 h post-infection of BMDMs (black bars). Mean ± s.e.m. of three independent experiments is shown. *P* values were calculated using Student's *t*-test.*$P < 0.05$; ***$P < 0.001$.



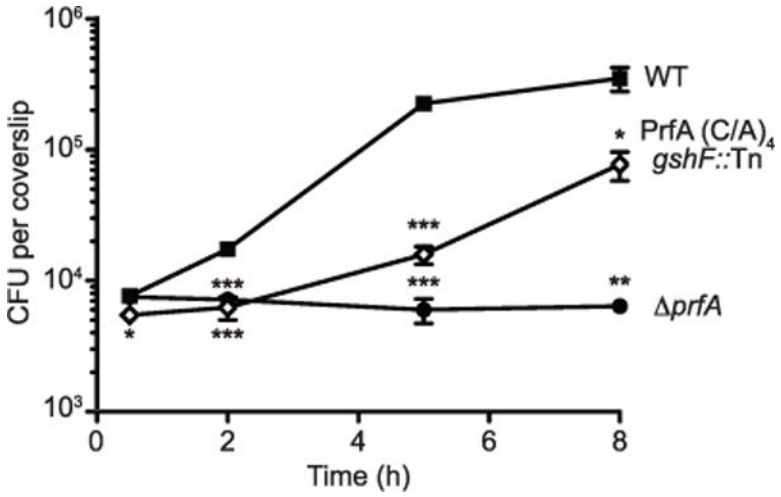
Extended Data Figure 4. Fluorescence polarization binding isotherms

a, Representative binding isotherms of wild-type PrfA plus DTT (circles), wild-type PrfA plus TCEP (squares), PrfA* (diamonds), and PrfA(C/A)$_4$ (crosses), to the PrfA box of *Phly*. **b**, Representative binding isotherms of wild-type PrfA plus DTT (circles), wild-type PrfA plus TCEP (squares), PrfA* (diamonds), PrfA(C/A)$_4$ (crosses), and oxidized wild-type PrfA (plus symbols), to the PrfA box of P*hly*. This plot underscores the very poor binding of oxidized wild-type PrfA to the PrfA box. In both panels the units of millipolarization (mP, *y* axis) have been normalized to allow the presentation of all binding isotherms on one graph. The protein concentration is shown in terms of protomer on the *x* axis.



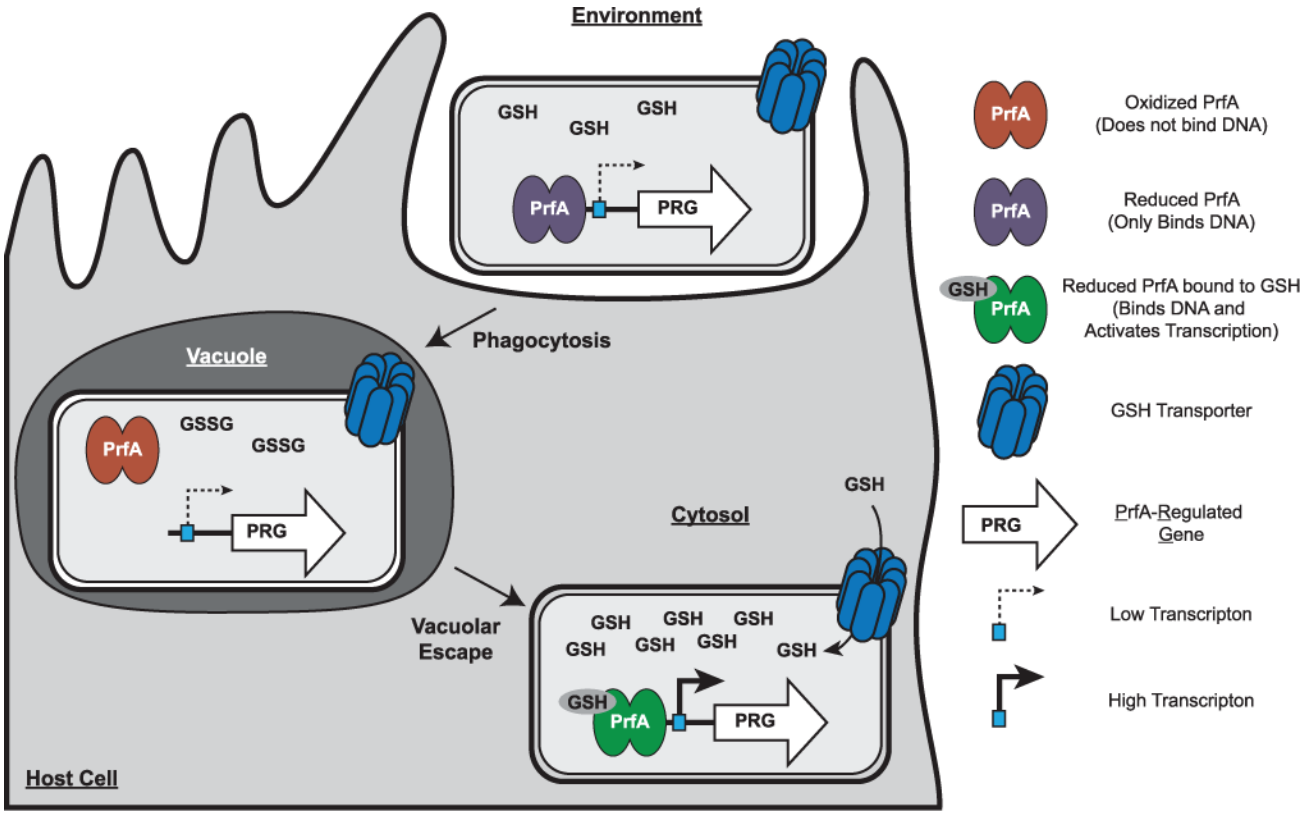
Extended Data Figure 5. PrfA(C/A)$_4$ expression in *L. monocytogenes* grown in broth

Immunoblot of PrfA in *L. monocytogenes* lysates harvested at early exponential phase in BHI. Mean ± s.e.m. of four independent experiments is shown.



Extended Data Figure 6. The PrfA(C/A)$_4$ *gshF::*Tn mutant exhibits a significant intracellular growth defect

The mean ± s.e.m. of four independent experiments is shown. *P* values were calculated using Student's *t*-test.; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.



Extended Data Figure 7. Model of glutathione-dependent PrfA activation

The process of infection or intercellular spread requires that *L. monocytogenes* inhabit an oxidizing vacuole, which may contain both reactive oxygen and nitrogen species. Upon oxidation, glutathione dimerizes to GSSG, which we have demonstrated does not bind PrfA. In addition, PrfA thiols may be reversibly oxidized, temporarily inactivating the protein by inhibiting DNA binding and leading to a downregulation of PrfA-regulated genes (PRG). *L. monocytogenes* could then enter the host cytosol, as PrfA activation is dispensable for vacuolar escape *in vivo*. The host cytosol is a highly reducing environment and upon entry into this compartment, all thiols are expected to be in the reduced form. In the absence of glutathione, it is likely that coenzyme A maintains redox homeostasis in the bacterium, as it is the most abundant LMW thiol in *L. monocytogenes*. Reduced glutathione could then bind PrfA and activate transcription of PRG. This two-step activation requirement may explain why the mechanism of PrfA activation has been a mystery for over two decades; the redox changes occurring during transit through a vacuole followed by replication in the highly reducing cytosol have yet to be recapitulated *in vitro*.

Extended Data Table 1

Strains and primers

Strain	*Escherichia coli*	Reference
NF-E1280	BL21(DE3) pET100.prfA	16
NF-E1281	BL21(DE3) pETIOO.prfA* (G145S)	16

Strain	***Escherichia coli***	**Reference**
DP-E6185	Rosetta pET100.prfA Quad	this work
Strain	***Listeria monocytogenes***	**Reference**
10403S	wt	[35]
DP-L3078	Δ*actA*	[36]
DP-L4317	Δ*prfA*	[37]
NF-L1177	PrfA* (G145S)	[16]
BH-3410	suicide	this work
DP-L6187	suicide *gshF::Tn*	this work
DP-L6188	Δ*gshF*	this work
DP-L6189	Δ*gshF* + *gshF*	this work
DP-L6190	PrfA*(G145S) Δ*gshF*	this work
DP-L6191	PrfA* (G145S) *gshF::Tn*	this work
NF-L1166	PrfA* (L140F)	[16]
DP-L6192	PrfA*(L140F)*gsnF::Tn*	this work
NF-L1214	PrfA* (Y63C)	[16]
DP-L6194	PrfA* (Y63C) *gshF::Tn*	this work
DP-L6195	PrfA $(C/A)_4$	this work
DP-L6196	PrfA $(C/A)_4$ *gshF::Tn*	this work
Primer	**Oligonucleotide**	**Amplicon**
MLR#123	CGACATAATATTTGCAGCGAC	*actA* for qPCR
MLR#124	TGCTTTCAACATTGCTATTAGG	
MLR#133	GACCCTAATCTCCGGAAGC	*gshF* for qPCR
MLR#134	TACAGAGTCAATCGAGTCCG	
MLR#121	GCGCAACAAACTGAAGCAAAG	*hly* for qPCR
MLR#122	CATTTGTCACTGCATCTCCG	
MLR#125	CTAACGGTCTATCTTCTAAGG	*hpt* for qPCR
MLR#126	CAATAATAATTGATATAATAGCGG	
MLR#150	ACCCTTGATTTTAGTTGCCAG	16S rRNA for qPCR
MLR#151	TGTGTAGCCCAGGTCATAAG	
MLR#102	GCTTCCAAGGAGCTAAAGAGGTCCCTAGCGCC	
MLR#103	CGGGGAATTTGTATCGATAAGGAATAGATTTAAAAATTTCGCTGTTATTTTG	Tn PCR Round#1
MLR#104	GGCCACGCGTCGACTAGTACNNNNNNNNNNCTTCT	Tn PCR Round#2
MLR#105	GGCCACGCGTCGACTAGTAC	
MLR#106	ACAATAAGGATAAATTTGAATACTAGTCTCGAGTGGGG	Tn Sequencing
KLH#1	TGAGGCATTAACATTTGTTAACGACGAT	*Phly* for DNA-binding assays
KLH#2	AACTGATTAACAAATGTTAGAGAAAACT	*PactA* for DNA-binding assays

References [16, 35–37] are cited in this table.

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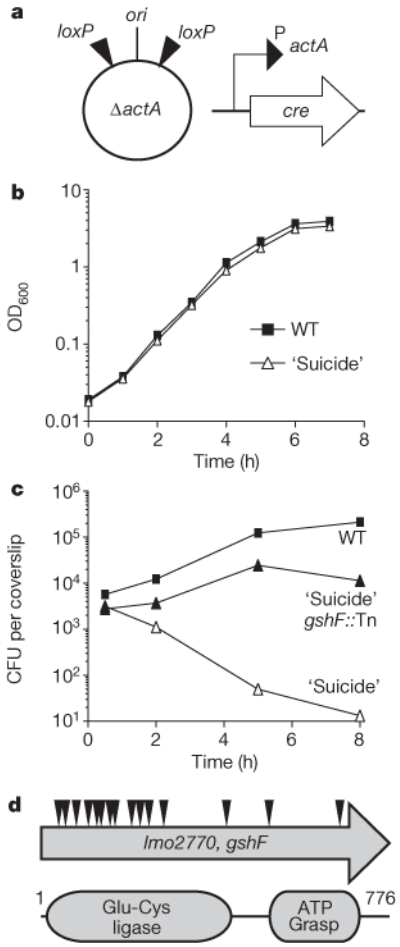

Figure 1. Forward genetic selection to identify factors required for virulence gene activation during infection

a, Schematic of the 'suicide' strain used for genetic selection. See text for description. **b**, Broth growth curve. Data are representative of three independent experiments. OD_{600}, optical density at 600 nm. **c**, BMDM growth curve. Data are a combination of three independent experiments. **d**, Schematic of transposon insertions identified in *gshF* and the conserved protein domains.

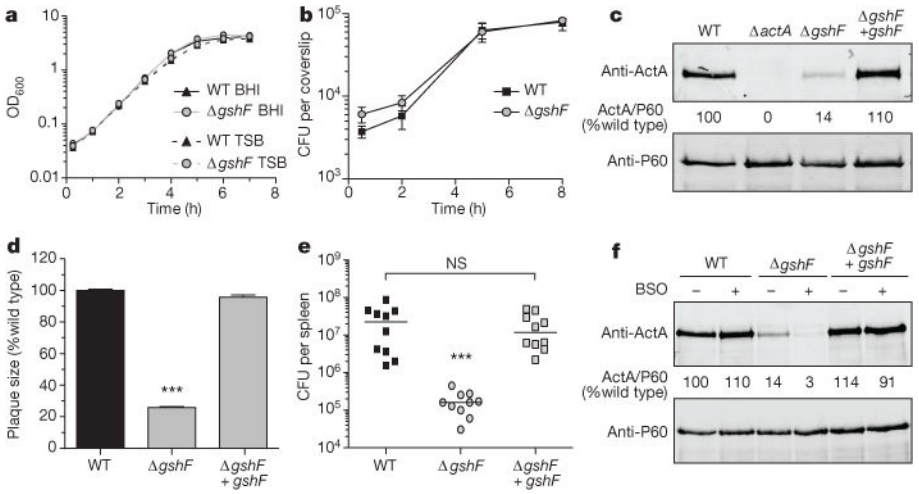

Figure 2. ***Listeria monocytogenes* Δ*gshF* is attenuated *in vivo***

a, Broth growth curve in brain heart infusion (BHI) or tryptic soy broth (TSB). Data are representative of three independent experiments. **b**, BMDM growth curve. Mean ± standard error of the mean (s.e.m.) for three independent experiments is shown. **c**, Representative immunoblot of infected BMDMs. Numbers are the mean of four independent experiments and indicate ActA normalized to P60, as a per cent of wild type. **d**, Plaque size. Mean ± s.e.m. for three independent experiments is shown. **e**, CD-1 mice were infected intravenously and analysed as described in Methods. Data are a combination of two independent experiments, $n = 10$ mice per strain. The median of each group is represented as a horizontal line. **f**, Representative immunoblot of infected BMDMs. Quantification is as described in **c**. In all panels P values were calculated using Student's t-test; $^{***}P < 0.001$; NS denotes $P > 0.05$.

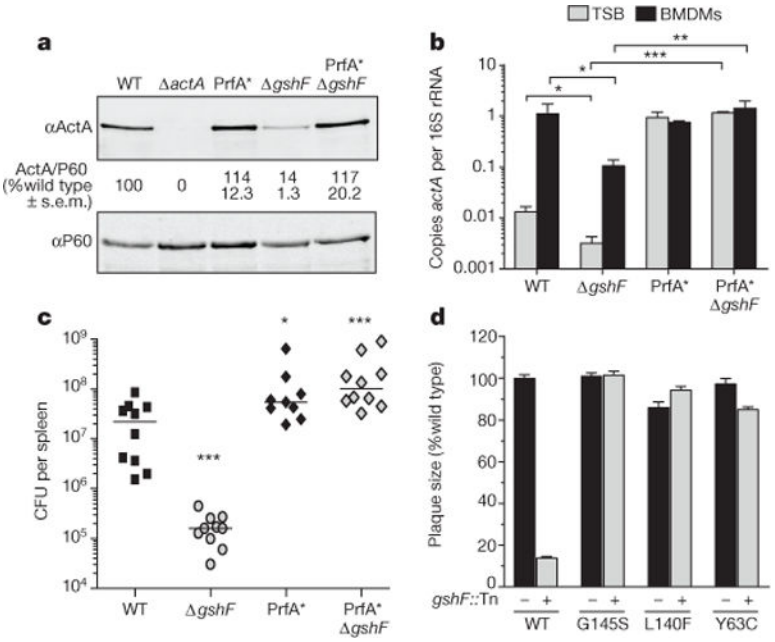

Figure 3. PrfA* bypasses the requirement for glutathione during infection

a, Representative immunoblot of infected BMDMs. Quantification is as described in Fig. 2. **b**, Quantitative reverse transcription polymerase chain reaction (RT–PCR) of *actA* transcript abundance. Mean ± s.e.m. for three independent experiments is shown. **c**, Mice were infected as described in Fig. 2. Data area combination of two independent experiments, n = 10 per strain. The median of each group is represented as a horizontal line. **d**, Plaque size. Mean ± s.e.m. for three independent experiments is shown. In all panels asterisks denote a significant difference compared to wild type, unless otherwise indicated, as determined by Student's *t*-test; *$P < 0.05$, **$P < 0.01$, ***$P<0.001$.

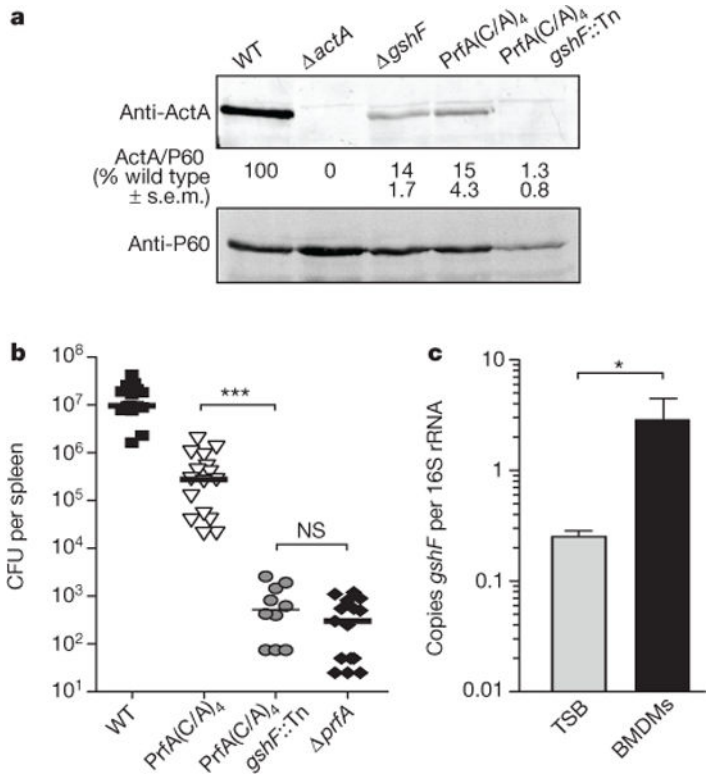

Figure 4. Glutathione-dependent PrfA activation is mediated by allosteric binding, not glutathionylation

a, Representative immunoblot of infected BMDMs. Quantification is as described in Fig. 2. **b**, Mice were infected as described in Fig. 2. Data are a combination of at least two independent experiments, $n = 10$ or 16 per strain. The median of each group is represented as a horizontal line. All strains were significantly different from wild type ($P < 0.001$). **c**, Quantitative RT–PCR of *gshF* transcript abundance. Mean ± s.e.m. for three independent experiments are shown. In all panels asterisks denote a significant difference compared to wild type, unless otherwise indicated, as determined by Student's *t*-test; $*P < 0.05$, $***P < 0.001$; NS denotes $P < 0.05$.

Table 1

DNA-binding and glutathione-binding affinities of PrfA

DNA-binding affinity (K_d ± s.e.m.)		
	P*hly* (nM)	**P*actA* (nM)**
Wild type (oxidized)	888.5 ± 140.3	ND
Wild type (reduced)	34.2 ± 4.9	96.4 ± 7.3
PrfA$(C/A)_4$	32.8 ± 5.5	124.9 ± 26.3
PrfA*	40.8 ± 3.3	45.4 ± 3.2

Glutathione-binding affinity (K_d ± s.e.m.)		
	GSH (mM)	**GSSG (mM)**
Wild type	4.37 ± 1.2	NBD
PrfA$(C/A)_4$	4.74 ± 1.5	NBD

DNA-binding affinity for the *hly* promoter (*Phly*) and the *actA* promoter (*PactA*), as measured by fluorescence anisotropy, and glutathione-binding affinity, as measured by bio-layer interferometry. The affinity of oxidized PrfA to P*actA* was not determined (ND). DNA-binding affinities of PrfA$(C/A)_4$ and PrfA* were unaffected by oxidation. For oxidized glutathione (GSSG) no measurable binding was detected (NBD).