Generation of a Metal-Responsive Transcriptional Regulator to Test What Confers Metal-Sensing in Cells

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Running title: Generation of a metal-sensor

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CAPSULE

Background: Metal-specific transcription has been correlated with the relative properties of a cells’ set of metal-sensors.

Results: One residue substitution enabled a DNA-binding formaldehyde-sensor to detect Zn(II) and cobalt.

Conclusion: Weaker DNA-affinity combined with tighter Zn(II)-affinity enabled Zn(II)-sensing with a smaller coupling free energy.

Significance: Relative affinity determined the best sensor in the set for Zn(II) but not for cobalt.

ABSTRACT

FrmR from Salmonella enterica serovar Typhimurium (a CsoR/RcnR-like transcriptional de-repressor) is shown to repress the frmRA operator-promoter and repression is alleviated by formaldehyde but not manganese, iron, cobalt, nickel, copper or Zn(II), within cells. In contrast, repression by a mutant FrmRE64H (which gains an RcnR metal-ligand) is alleviated by cobalt and Zn(II). Unexpectedly, FrmR was found to already bind Co(II), Zn(II) and Cu(I) and moreover metals, as well as formaldehyde, trigger an allosteric response which weakens DNA affinity. However, the sensory metal sites of the cells’ endogenous metal sensors (RcnR, ZntR, Zur and CueR) are all tighter than FrmR for their cognate metals. Furthermore, the endogenous metal sensors are shown to out-compete FrmR. The metal-sensing FrmRE64H mutant has tighter metal-affinities than FrmR by approximately one order of magnitude. Gain of cobalt-sensing by FrmRE64H remains enigmatic since the cobalt affinity of FrmRE64H is substantially weaker than that of the endogenous cobalt sensor. Cobalt-sensing requires glutathione which may assist cobalt-access, conferring a kinetic advantage. For Zn(II) the metal affinity of FrmRE64H approaches the metal affinities of cognate Zn(II) sensors. Counter-intuitively, the allosteric coupling free energy for Zn(II) is smaller in metal-sensing FrmRE64H compared to non-sensing FrmR. By determining the copies of FrmR and FrmRE64H tetramers per cell, then estimating promoter occupancy as a function of intracellular Zn(II) concentration, we show how a modest tightening of Zn(II) affinity, plus weakened DNA-affinity of the apo-protein, conspire to make the relative properties of FrmRE64H (compared to ZntR and Zur) sufficient to sense Zn(II) inside cells.

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INTRODUCTION

Metal-sensing, DNA-binding, transcriptional-regulators are central to the machinery which optimises buffered metal-concentrations inside cells to enable correct protein-metallation (1, 2). In general, the tighter $K_{\text{metal}}$ of a metal-sensor the lower [buffered metal] (1). Fresh experimental approaches are needed to test hypotheses about the mechanisms determining which metal(s) each sensor detects. Uncertainty also remains about the nature of the exchangeable pools of different metals including the major ligands, the precise buffered metal-concentrations, and how these vary under different environmental conditions or between organisms.

Metal sensors tend to bind divalent metals with an order of affinity which matches the Irving-Williams series, regardless of which metal(s) they detect in a cell (1-3). This raises questions about how a sub-set of sensors can detect the weaker binding metals in vivo (4-6). One facet of the solution is that the kinetics of access to different metals can vary from sensor to sensor, for example due to interactions with specific donor molecules, including metallochaperones (1, 6-8). Another part of the solution is that the allosteric mechanism connecting metal-binding to DNA-binding can be metal-selective (9-12). Thus, a weaker binding metal can nonetheless be more effective at triggering the conformational changes which alter gene expression (10, 13). For metal-dependent de-repressors and co-repressors the coupling free energy, $\Delta G_{C}^{\text{metal-sensor\cdotDNA}}$, is typically larger for more effective metals (9). Unexpectedly, here we see how a metal can also become effective without increasing $\Delta G_{C}^{\text{metal-sensor\cdotDNA}}$; if $K_{\text{DNA}}$ of the apo-form of a de-repressor is suitably weakened, to confer two mechanistic advantages in favour of Zn(II)-detection. Contrary to general dogma, here $\Delta G_{C}^{\text{Zn(II)-sensor\cdotDNA}}$ is smaller in the Zn(II)-sensing relative to the non-sensing wild-type protein.

In the course of a collaborative programme to characterise the complement of metal-sensors from Salmonella enterica serovar Typhimurium strain SL1344 (hereafter referred to as Salmonella) we identified two genes encoding proteins with sequence similarity to members of the CsoR/RcnR-family of DNA-binding, metal-responsive, transcriptional de-repressors (14-17). These are now shown to be Salmonella homologues of RcnR and FrmR. RcnR in Escherichia coli responds to cobalt and nickel while CsoR, first discovered in Mycobacterium tuberculosis, responds to Cu(I) (15-17). Related metal-sensors characterised from other bacteria detect the same metals (18-27). Additionally, two homologues have been identified that respond to effectors other than metals, namely CstR from Staphylococcus aureus which detects persulfide, plus E. coli FrmR (28-30). CsoR forms a three helix bundle which assembles into tetramers (15). The sensory Cu(I) site exploits a conserved Cys-thiolate from the N-terminal end of helix $\alpha 2$ of one sub-unit in combination with an HxxxC motif from within helix $\alpha 2'$ of a second sub-unit (15) (Fig. 1A). Three ligands in similar locations (with HxxxC replaced by HxXxH along with additional ones from the N-terminal region of helix $\alpha 1'$, are recruited to the sensory metal-site of RcnR (Fig. 1B) (17, 31, 32). A single residue variant of E. coli RcnR (H3E) also responds to Zn(II) (31).

In a global screen to discover the consequences of reading-through amber stop codons, E. coli FrmR (which has such a stop) emerged as the transcriptional repressor of the frmRAB operon (30). FrmA has formaldehyde dehydrogenase activity and the operon was subsequently shown to respond to exogenous formaldehyde (30, 33). This operon is de-repressed during anaerobic respiration using trimethylamine-N-oxide (TMAO) as the terminal electron acceptor where endogenous formaldehyde is generated as a by-product of TMAO demethylation (34). CO-releasing molecules and chloride treatments also trigger expression of the frm operon (35, 36). There are no published studies of the Salmonella FrmR homologue. At least two potential metal-ligands are retained in Salmonella FrmR namely C at the N-terminus of helix $\alpha 2$ but HxXxE (rather than HxXxH of paralogous Salmonella RcnR) at helix $\alpha 2'$ (Fig. 1A and B). Despite sequence similarity between FrmR and other CsoR/RcnR family members, whether or not (any) FrmRs de-repress gene expression in response to metals, remains untested.

Recent studies have shown that relative-affinity, relative-allostery and relative-access determine the ability of metal-sensors to respond selectively in vivo (1). This is exemplified by comparing metal-affinities ($K_{\text{metal}}$) and metal-responsive allostery ($\Delta G_{C}^{\text{metal-sensor\cdotDNA}}$) among multiple metal-sensors, and for multiple metals in Synechocystis PCC 6803 (1, 6, 11, 18). Thus, InrS responds to nickel in vivo and has a $K_{\text{Ni(II)}}$ which is
substantially tighter than \( K_{\text{Ni(II)}} \) of cobalt-sensing CoaR and Zn(II)-sensing ZiaR or Zur (a representative from each family of metal-sensors present in this organism) (18). Provided the distribution of Ni(II) follows thermodynamic equilibrium predictions, as the [Ni(II)] rises, InrS will be the first to respond, de-repressing its target gene ziaA (encoding a Zn(II)-efflux ATPase). In contrast, cobalt-sensing does not correlate with relative affinity and CoaR has the weakest \( K_{\text{Co(II)}} \) in the set of sensors (6). There is evidence that the cobalt effector may be preferentially channelled to CoaR, and thus relative access has been invoked as the explanation for selective detection of cobalt (6). In summary, it is hypothesised that the sensor which is triggered by a metal is simply the most responsive within a cells’ set of sensors, based upon relative-affinity, relative-allostery and relative-access (1). This hypothesis is now tested via a mutation conferring gain-of-metal-sensing.

Here, the Co(II)-, Zn(II)-, and Cu(I)-binding affinities of Salmonella FrmR are determined, and compared to equivalent data for the cognate sensors of these metals, namely Salmonella homologues of RcnR, Zn(II)-sensing ZntR and Zur, and Cu(I)-sensing CueR. FrmR is found not to sense metals within cells yet an E64H substitution (creating a Salmonella RcnR-like helix \( \alpha' \) HxxxH motif) gains responsiveness to cobalt and Zn(II) \textit{in vivo}. By comparing the biochemical properties of Salmonella FrmR with FrmRE64H, and then relating these parameters to endogenous sensors for cobalt, Zn(II) and Cu(I), relative-properties which, in combination, enable metal-sensing are identified.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and DNA manipulations** - \textit{S. enterica} sv. Typhimurium strain SL1344 was used as wild type and strain LB5010a was used as a restriction-deficient modification-proficient host for DNA manipulations: Both were a gift from J.S. Cavet (University of Manchester). \textit{E. coli} strain DH5\(\alpha\) was used for routine cloning. Bacteria were cultured with shaking at 37 °C in Luria-Bertani (LB) medium or M9 minimal medium (37), supplemented with thiamine (0.001% w/v) and L-histidine (20 μg ml\(^{-1}\)). Carbenicillin (100 μg ml\(^{-1}\)), kanamycin (50 μg ml\(^{-1}\)), and/or chloramphenicol (10 μg ml\(^{-1}\)) were added where appropriate. Cells were transformed to antibiotic resistance as described (37, 38). All generated plasmid constructs were checked by sequence analysis. Primers are listed in supplemental table S1.

**Generation of Salmonella deletion mutants** - Deletion derivatives of strain LB5010a were obtained using the \( \lambda \) Red method (38) using plasmid pKD3 and primers 1 and 2 for \( \text{frmRA} \) or 3 and 4 for \( \text{gshA} \). Mutagenesis was performed using strain LB5010a and selection of mutants achieved using LB medium supplemented with chloramphenicol. Mutations were subsequently moved to SL1344 or derivatives using P22 phage transduction and validated by PCR using primers 5 and 6 for \( \text{frmR} \) or 7 and 8 for \( \text{gshA} \). The antibiotic-resistance cassette from the \( \Delta\text{frmR}::\text{cat} \) locus was removed using the helper plasmid pCP20 carrying the FLP recombinase.

**Generation of promoter-lacZ fusion constructs and β-galactosidase assays** - \( P_{\text{frmRA}} \) or \( P_{\text{frmRE64H}} \) were amplified from SL1344 genomic DNA using primer 9, and either primer 10 (for \( P_{\text{frmRA}} \)) or 11 (for \( P_{\text{frmRE64H}} \)) and ligated into pGEM-T. Site-directed mutagenesis to generate \( P_{\text{frmRA}}\cdot\text{frmRE64H} \) and \( P_{\text{frmRA}}\cdot\text{frmRE64H}^{\text{DOWN}} \) was conducted via the ‘QuikChange’ protocol (Stratagene) using pGEM- \( P_{\text{frmRA}}\cdot\text{frmRE64H} \) as template and primers 12-23. Codon optimisation of the \( \text{frmRE64H} \) coding region to generate \( P_{\text{frmRA}}\cdot\text{frmRE64H}^{\text{OPP}} \) (supplemental table S2) was achieved using GeneArt Gene Synthesis (Life Technologies) and optimisation for \textit{Salmonella} Typhimurium. The \( \text{rcnR}-\text{P}_{\text{rcnA}} \) region was amplified from SL1344 genomic DNA using primers 24 and 25. Digested fragments were cloned into the Smal/BamHII site of pRS415 (39). \( P_{\text{rcnA}} \) cloned into pRS415 was provided by J.S. Cavet (University of Manchester). The resulting constructs were introduced into strain LB5010a prior to strain SL1344. β-galactosidase assays were performed as described (40) in triplicate on
at least three separate occasions. Overnight cultures were grown in M9 minimal medium, diluted 1:50 in fresh medium supplemented with maximum non-inhibitory concentrations (MNIC; defined as the maximum concentration which inhibited growth by ~10%) of metals, formaldehyde, ethylenediaminetetraacetic acid (EDTA) or N,N,N’,N’-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) and grown to mid-logarithmic phase prior to assays. For time-course experiments, cells were grown to early-logarithmic phase, statically cooled to 25 °C for 20 min followed by 2 h incubation in the presence of metal or formaldehyde. The metal salts used were MnCl₂, C₆H₅FeO₇, CoCl₂, NiSO₄, CuSO₄, ZnSO₄ and concentrations were verified by ICP-MS. MNICs under these growth conditions were 200 μM MnCl₂, 5 μM CoCl₂, 20 μM NiSO₄, 25 μM CuSO₄, 50 μM ZnSO₄, 50 μM formaldehyde, 25 μM EDTA and 0.25 μM TPEN, with the exception that 1 μM CoCl₂ was found to be the MNIC for cells expressing rcnR-P<sub>rend</sub>. Addition of C₆H₅FeO₇ improved growth and 5 μM was used throughout.

**Protein expression and purification** - The frmR, zntR, zur, and rcnR coding regions were amplified from SL1344 genomic DNA using primers 26-33 and ligated directly into pET29a (Novagen) (or via pGEM-T) using the NdeI/BamHI site for frmR, zntR and rcnR or NdeI/EcoRI site for zur. Site-directed mutagenesis was conducted as described above using template pETfrmR and primers 34 and 35 to generate pETfrmRE64H or template pETzntRLT2 and primers 36 and 37 to generate pETzntR. Proteins were expressed in exponentially growing *E. coli* BL21(DE3) for 3 h at 37 °C using 0.2 mM 1-thio-β-d-galactopyranoside (IPTG). The medium was supplemented with 50 μM ZnSO₄ for Zur (to promote metallation of the structural zinc site). Harvested BL21(DE3) pETfrmR or pETfrmRE64H cells were resuspended in Buffer A (300 mM NaCl, 5 mM imidazole, 5 mM DTT, 50 mM sodium phosphate pH 7.4) with addition of 1 mM PMSF and following sonication and clarification, applied to an equilibrated 5 ml HiTrap Heparin column (GE Healthcare), washed in the same buffer and eluted in a single step using Buffer B with 800 mM NaCl. Proteins were further purified by size-exclusion chromatography (HiLoad 26/60 Superdex 75, GE Healthcare) equilibrated in 300 mM NaCl, 10 mM DTT, 10 mM EDTA, 10 mM HEPES pH 7.8 for FrmR and FrmRE64H, 50 mM NaCl, 5 mM DTT, 1 mM EDTA, 10 mM HEPES pH 7.0 for ZntR, 300 mM NaCl, 5 mM DTT, 1 mM EDTA, 10 mM HEPES pH 7.8 for Zur, and Buffer B for RcnR. FrmR and FrmRE64H were diluted to 100 mM NaCl, 10 mM DTT, 10 mM EDTA, 10 mM HEPES pH 7.8, applied to an equilibrated 1 ml HiTrap Heparin column (GE Healthcare) and washed with 10 column volumes of the same buffer. ZntR and Zur were treated in the same way, except using 5 mM NaCl, 1 mM EDTA, 5 mM DTT, 10 mM HEPES pH 7.0 for ZntR, and 100 mM NaCl, 5 mM DTT, 1 mM EDTA, 10 mM HEPES pH 7.8 for Zur. FrmR, FrmRE64H, ZntR and Zur were eluted in a single step using respective binding buffers plus 500 mM NaCl. RcnR was diluted to 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 10 mM HEPES pH 7.0 and applied to an equilibrated 5 ml HiTrap SP column (GE Healthcare), washed in the same buffer plus 200 mM NaCl, and eluted in 300 mM NaCl. CueR was expressed and purified as described previously (41). Anaerobic protein stocks were prepared by applying purified protein to a pre-equilibrated 1 ml HiTrap Heparin column (diluting FrmR, FrmRE64H, ZntR and Zur as described above, and without dilution of RcnR). The protein-loaded column was moved into an anaerobic chamber, washed with >10 column volumes of chelex treated, N₂-purged 80 mM KCl, 20 mM NaCl, 10 mM HEPES pH 7.0 for FrmR, FrmRE64H and Zur, 4 mM KCl, 1 mM NaCl, 10 mM HEPES pH 7.0 for ZntR, or 240 mM KCl, 60 mM NaCl, 10 mM HEPES pH 7.0 for RcnR. Proteins were eluted in a single step using 400 mM KCl, 100 mM NaCl, 10 mM HEPES pH 7.0 for FrmR, FrmRE64H, Zur and ZntR or 800 mM KCl, 200 mM NaCl, 10 mM HEPES pH 7.0 for RcnR. Proteins were quantified by measurement of A<sub>280 nm</sub> and using experimentally determined extinction coefficients obtained via quantitative amino acid analysis (Abingdon Health Laboratory).
copper-binding by the Sephadex matrix.

suggests (at least) some competition from and
during experimentation with FrmR or FrmRE64H,
concentrations of FrmR, FrmRE64H or Zur as
MS and protein by Bradford assay using known
GE Healthcare) in the same buffer conditions.

exclusion chromatography (PD-10 Sephadex G25,
and an aliquot (0.5 ml) was resolved by size-
NaCl, 400 mM KCl and 10 mM HEPES pH 7.0
[> 95% Cu(I)] or EDTA (as stated) in 100 mM
Perkin Elmer λ35 UV
absorbance spectra recorded at equilibrium using a
ZnCl
Precipitation of ZntR was observed with further
incubation (60 min) with an excess of ZnCl
2
2
, CuCl and ZnCl
2
were carried out in 100 mM NaCl, 400 mM KCl
and 10 mM HEPES pH 7.0, with addition of
5% (v/v) glycerol for RcnR. Concentrations of metal stocks
(CoCl
2
, NiCl
2
, CuCl and ZnCl
2
) were verified by inductively coupled plasma-mass spectrometry
(ICP-MS). CuCl was prepared as described previously and confirmed to be > 95% Cu(I) by
inductively coupled plasma-mass spectrometry
(inductively coupled plasma-mass spectrometry (Agilent Technologies), as
described previously (6). Fura-2 was quantified
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UV-visible absorption spectroscopy- Experiments
were carried out in 100 mM NaCl, 400 mM KCl
and 10 mM HEPES pH 7.0 for FrmR, FrmRE64H,
ZntR, Zur and CueR, with inclusion of 5 % (v/v)
glycerol for RcnR. Concentrations of metal stocks
(CoCl
2
, NiCl
2
, CuCl and ZnCl
2
) were verified by inductively coupled plasma-mass spectrometry
(ICP-MS). CuCl was prepared as described previously and confirmed to be > 95% Cu(I) by
titration against bathocuproine sulfonate (BCS)
(42). CoCl
2
, NiCl
2
or CuCl [ > 95% Cu(I)] were
titrated into protein, or ZnCl
2
was titrated into
protein pre-equilibrated with CoCl
2
, and the
absorbance spectra recorded at equilibrium using a
Perkin Elmer λ35 UV-visible spectrophotometer. Precipitation of ZntR was observed with further
ZnCl
2
additions to Co(II)-ZntR than those shown.

Protein-metal migration by size-exclusion
chromatography - FrmR, FrmRE64H or Zur were
incubated (60 min) with an excess of ZnCl
2
, CuCl
[ > 95% Cu(I)] or EDTA (as stated) in 100 mM
NaCl, 400 mM KCl and 10 mM HEPES pH 7.0
and an aliquot (0.5 ml) was resolved by size-
exclusion chromatography (PD-10 Sephadex G25,
GE Healthcare) in the same buffer conditions.
Fractions (0.5 ml) were analysed for metal by ICP-
MS and protein by Bradford assay using known
centrations of FrmR, FrmRE64H or Zur as
standards. Failure to recover all of the copper
during experimentation with FrmR or FrmRE64H,
suggests (at least) some competition from and
copper-binding by the Sephadex matrix.

Protein-chelator-Zn(II) competitions- Experiments
were carried out in 100 mM NaCl, 400 mM KCl
and 10 mM HEPES pH 7.0 as described
previously (11). ZnCl
2
was titrated into a mixed
solution of protein and mag fura-2 or protein and
quin-2 and absorbance was recorded at
equilibrium at 366 nm (mag fura-2) and 261 nm or
265 nm (quin-2). Data were fit to the models
described in Figure legends and Table 1 footnotes
using Dynafit (43) to determine Zn(II) binding
constants. Mag fura-2 and quin-2 were quantified
using extinction coefficients \( \varepsilon_{369 \text{ nm}} = 22000 \text{ M}^{-1} \text{ cm}^{-1} \) (44) and \( \varepsilon_{261 \text{ nm}} = 37000 \text{ M}^{-1} \text{ cm}^{-1} \) (45),
respectively. \( K_{\text{Zn(II)}} = 2 \times 10^8 \text{ M} \) for mag-fura-2 at
pH 7.0 (46) and \( K_{\text{Zn(II)}} = 3.7 \times 10^{12} \text{ M} \) at pH 7.0 for
quin-2 (45).

Protein-chelator-Co(II) competitions- CoCl
2
was
titrated into a mixed solution of protein and fura-2,
or protein and Bis-Tris in 100 mM NaCl, 400 mM
KCl, and 10 mM HEPES pH 7.0, with addition of
5% (v/v) glycerol for experiments with RcnR. For
competition with fura-2, fluorescence emission
was recorded at equilibrium at 510 nm (\( \lambda_{\text{ex}} = 360 \text{ nm} \); \( T = 20 \degree \text{C} \)) using a Cary Eclipse Fluorescence
Spectrophotometer (Agilent Technologies), as
described previously (6). Fura-2 was quantified
using the extinction coefficient \( \varepsilon_{363 \text{ nm}} = 28000 \text{ M}^{-1} \text{ cm}^{-1} \) (6). For competition with Bis-Tris,
absorbance spectra were recorded at equilibrium.
Data were fit to the models described in Figure
legends and Table 1 footnotes using Dynafit to
determine Co(II) binding constants (43). \( K_{\text{Co(II)}} =
8.64 \times 10^9 \text{ M} \) for fura-2 at pH 7.0 (47), and \( K_{\text{Co(II)}} =
2.26 \times 10^{12} \text{ M} \) at pH 7.0 for Bis-Tris using the
absolute formation constant for Co(II)-Bis-Tris
and Schwarzenbach’s \( \alpha \)-coefficient method (48).

Protein-chelator-Cu(I) competitions- Experiments
were carried out in 100 mM NaCl, 400 mM KCl
and 10 mM HEPES pH 7.0. CuCl [ > 95% Cu(I)]
was titrated into a mixed solution of protein and
BCA and the absorbance at 562 nm was recorded
in equilibrium. Data were fit to the models
described in Figure legends and Table 1 footnotes
using Dynafit to determine Cu(I) binding constants (43). \( K_{\text{Cu(I)}} = 1.58 \times 10^{17} \text{ M}^{-2} \) at pH 7.0 for BCA
(48). For BCS, the absorbance at 483 nm was
recorded following titration with CuCl (to generate
a calibration curve), or following pre-incubation of
BCS with CuCl (10 min) and addition of CueR.
The absorbance at 483 nm was monitored to
equilibrium. $K_{\text{Cu(I)}}$ of the tightest site of CueR was calculated using the following equation (48):

$$K_{\text{Cu(I)}} = \frac{[P]_{\text{total}} - [M]_{\text{total}} - [L]_{\text{total}}}{[M]_{\text{total}} - [L]_{\text{total}} + [P]_{\text{total}} - [M]_{\text{total}} - [L]_{\text{total}} - 1}$$

CueR is expected to be a dimer with two metal-binding sites (49) which bind Cu(I) with negative co-operativity (41), therefore, the concentration of the tightest metal binding site, [P]_{total}, was taken as [CueR monomer] × 0.5. [L]_{total} is the total [BCS]. The absorbance at 483 nm at the end point of competition between CueR and BCS was used to calculate [Cu(I)BCS$_2$], [ML$_2$], from the slope of the calibration curve. Assuming all Cu(I) is bound to either CueR or BCS, the concentration of Cu(I)-CueR, [MP], was determined by subtracting [ML$_2$] from total metal, [M$_{\text{total}}$], used in the competition. $eta_{\text{2Cu(I)}} = 6.01 \times 10^{19} \text{ M}^{-2}$ at pH 7.0 for BCS using the absolute formation constant of Cu(I)-BCS and Schwarzenbach’s $\alpha$-coefficient method (48).

**Fluorescence spectroscopy** - Experiments were carried out in 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0. ZnCl$_2$ was titrated into ZnR, and fluorescence emission spectra ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 303$ nm, $T = 20$ °C) recorded at equilibrium using a Cary Eclipse Fluorescence Spectrophotometer. Precipitation was observed with addition of more than 1.1 molar equivalents ZnCl$_2$.

**Interprotein metal-exchange** - For competition of FrmR with CueR or ZntR, FrmR (40 μM, monomer) was equilibrated with 10 μM CuCl (> 95% Cu(I)) or ZnCl$_2$, in 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0 before addition of either 20 μM (monomer) CueR or ZntR, respectively. Protein mixtures (1 ml) were diluted to 20 mM NaCl, 80 mM KCl, 10 mM HEPES pH 7.0 and applied to a heparin affinity chromatography column. FrmR and CueR were differentially eluted in 60 mM NaCl, 240 mM KCl, 10 mM HEPES pH 7.0. ZntR does not bind the column, and FrmR was eluted with 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0. Fractions (1 ml) were assayed for metal by ICP-MS and protein by SDS-PAGE. For competition of FrmR with RcnR, apo-subtracted difference spectra were taken at equilibrium of FrmR or RcnR incubated with 9.9 μM CoCl$_2$, or after addition of RcnR to Co(II)-FrmR (using the same concentrations as control). Buffer conditions were 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0.

**Protein quantification by liquid chromatography-tandem mass spectrometry** - Cellular lysates were prepared from logarithmic cultures grown in M9 minimal medium. Cell number was determined by enumeration on LB agar plates. Harvested cells were resuspended in 40 mM NaCl, 160 mM KCl, 10 mM EDTA, 10 mM DTT, 10 mM HEPES pH 7.8 with addition of protease inhibitor cocktail (Sigma) and post-sonication, the soluble cell lysate was syringe filtered (0.45 μm pore size), snap frozen in liquid N$_2$, stored at -80 °C, and thawed on ice before use. Total protein was determined by the Bradford assay, using BSA as a standard. Purified stocks of FrmR or FrmRE64H were quantified by amino acid analysis (Proteomics Core Facility, University of California), stored at -80 °C and thawed on ice before dilution in PBS to 0.6 mg ml$^{-1}$. For standard curves, proteins were further diluted in soluble cell lysates from ΔfrmR cells to generate standard curve concentrations of 5, 10, 50, 250, 425 and 500 ng 100 μl$^{-1}$ (which defined the limits for quantification). Aliquots were stored at -80 °C. Working internal standards were prepared by dilution of labelled peptides GQVEALER$^{[13]C_6,15N_4}$, DELVSGETTPDQR$^{[13]C_6,15N_4}$, and DHLVSGETTPDQR$^{[15]C_6,15N_4}$ (arginine residues labelled) (Thermo Fisher) in 15% (v/v) acetonitrile with 0.1% (v/v) formic acid solution to obtain final concentrations of 313 fmol μl$^{-1}$ of each peptide. For experimental samples (soluble lysates from ΔfrmR cells to generate standard curve constructs and variants) and standard curve samples, 100 μl was precipitated using 300 μl methanol (mixing at 2000 rpm for 1 min) before centrifugation (900 x g, 5 min, room temperature). Pellets were suspended in 400 μl 200 mM NH$_4$HCO$_3$ in 10% (v/v) methanol (mixing at 2000 rpm for 10 min) and 10 μl internal standard added. Pellet digestion was performed with 10 μl trypsin (14 mg ml$^{-1}$) and mixing (1000 rpm, 37°C, 16 h) and stopped with 10 μl 15% (v/v) formic acid. The digested samples were centrifuged (6000 x g for 5 min at room temperature) to remove particulate material. Solvent was removed from clarified supernatants (50-100 μl) using a centrifugal evaporator (Thermo Scientific SpeedVac system). Samples were separated by gradient elution at 0.3 ml min$^{-1}$ using a Zorbax Eclipse Plus C18 column.
Determination of intracellular glutathione and glutathione volume of 1 fl.

Intracellular glutathione was calculated using a cell incubation in the absence or presence of MNIC cooled to 25 °C for 20 min followed by 30 min and grown to early-logarithmic phase, statically minimal medium, diluted 1:50 in fresh medium prepared from overnight cultures grown in M9 glutathione assay kit (Sigma) according to GQVEALER in all validation runs.

Determining intracellular glutathione-

Intracellular glutathione was measured using a glutathione assay kit (Sigma) according to manufacturers’ instructions. Cellular lysates were prepared from overnight cultures grown in M9 minimal medium, diluted 1:50 in fresh medium and grown to early-logarithmic phase, statically cooled to 25 °C for 20 min followed by 30 min incubation in the absence or presence of MNIC ZnSO₄. Viable cells were enumerated on LB agar and [glutathione] was calculated using a cell volume of 1 fl.

Fluorescence anisotropy-

Complementary single stranded oligonucleotides 38 (HEX-labelled) and 39 (containing the identified FrmR binding site and flanking nucleotides, Fig. 1C) were annealed by heating 10 or 200 μM of each strand in 10 mM HEPES, pH 7.0, 150 mM NaCl to 95 °C and cooled to room temperature overnight. For protein:DNA stoichiometry experiments, the fluorescently labelled, annealed probe (designated frmRAPro) was diluted to 2.5 μM in 10 mM HEPES, pH 7.0, 60 mM NaCl, 240 mM KCl and 5 mM EDTA and titrated with FrmR or FrmRE64H prepared in 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0 and 5 mM EDTA. For KＤＤＡＮＡ determination, frmRAPro was diluted to 10 nM, with addition of 5 mM EDTA or 5 μM ZnCl₂ as required. FrmR or FrmRE64H were prepared as above with inclusion of 5 mM EDTA or 1.2 molar equivalents of ZnCl₂ or CuCl (≥ 95% Cu(I)) as appropriate. Changes in anisotropy (Δrobs) were measured using a modified Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) fitted with polarising filters (λex = 530 nm, λem = 570 nm, averaging time = 20 s, replicates = 5, T = 25 °C) as described previously (11). Upon each addition, the cuvette was allowed to equilibrate for 5 min before recording data. Data were fit to the model described in the Figure legends and Table 2 footnotes using Dynafit (43). For experiments with Cu(I)- or Zn(II)-FrmR or FrmRE64H, where DNA binding did not saturate, the average fitted Δrobs maximum value from apo-protein experiments was used in the script. The coupling free energy ΔGΔ, linking DNA binding to metal binding, was calculated as described previously (11) using: ΔGΔ = −RΔH/KT, where R = 8.314 J K⁻¹ mol⁻¹ (gas constant), T = 298.15 K (temperature at which experiment was conducted), and KΔ = KΔDNA-metal-protein/KΔDNA-apo-protein (9). Mean ΔGΔ values (and standard deviations) were calculated from the full set of (equally weighted) possible pair-wise permutations of KΔ.

Fractional occupancy models-

Fractional occupancy of the tightest metal binding site of a sensor with metal as a function of buffered [metal], was determined using: (θ) = [Metal]buffered/(Kmetal + [Metal]buffered). Kmetal = KD (tightest site) of sensor for metal, experimentally determined (Kmetal_sensor) (Table 1) (48). For FrmR (and variants), Kmetal was additionally calculated for the DNA-bound form (Kmetal_sensor·DNA) from the coupling constant, (KΔ) (Fig. 10E). The concentration of apo- and Zn(II)-protein at a given [Zn(II)] was calculated using the number of tetramers per cell (FrmR and variants; Fig. 9K), and a cell volume of 1 fl. Fractional DNA occupancies with apo- and Zn(II)-protein over a range of protein concentrations were modelled using Dynafit (43) (1:1 binding of tetramer:DNA; assuming the binding of one tetramer conferred repression) with KΔDNA (from Table 2) and [PfrmRA] as fixed parameters (sample Dynafit script is also shown in the supplemental material). [PfrmRA] was calculated assuming 15 copies cell⁻¹ (due to the presence on low-copy number reporter plasmid) and a cell volume of 1 fl. The response was set at 1/[PfrmRA]. The fractional occupancy of PfrmRA with apo- and Zn(II)-protein was summed to give fractional occupancy of PfrmRA at any given buffered [Zn(II)].

RESULTS

Generation of a metal-sensor
CsoR/RcnR-like repressor FrmR solely detects formaldehyde and not metals- Despite similarity between FrmR and metal-sensing transcriptional de-repressors, exposing Salmonella cultures to maximum non-inhibitory concentrations (MNIC) of MnCl₂, CaH₂FeO₇, CoCl₂, NiSO₄, CuSO₄ or ZnSO₄, does not de-repress from P₉₅R-frmR fused to lacZ in ΔfrmR cells (Fig. 2A). Exposure to MNIC of formaldehyde does de-repress expression from P₉₅R (Fig. 2B and C). Thus, in common with E. coli FrmR (30), the Salmonella homologue represses expression from the frmRA operator-promoter with repression alleviated by formaldehyde, and here we show that repression by Salmonella FrmR is not alleviated by metals.

Substitution of FrmR E64 for an RcnR metal-ligand confers Zn(II) and cobalt detection in cells- Replacement of FrmR residue 64 (glutamate) with histidine (a metal-ligand in RcnR; Fig. 1A and B), generates a metal-sensing variant of FrmR (Fig. 2D). Repression is alleviated by CoCl₂ and ZnSO₄ in ΔfrmR cells containing P₉₅R-frmRE64H (but not P₉₅R-frmR) fused to lacZ (Fig. 2D and E). MnCl₂, CaH₂FeO₇, NiSO₄ and CuSO₄, did not affect expression from P₉₅R-frmRE64H (or P₉₅R-frmR) while formaldehyde-responsiveness was retained. Notably, metal-responsive family members RcnR and CsoR do respond to nickel and copper (15-17). In summary, a single residue change which mimics the metal-sensing site of RcnR is sufficient to create a detector of cellular Zn(II) and cobalt.

FrmRE64H and FrmR both bind Co(II), Cu(I) and Zn(II)- It was anticipated that the introduced histidine residue created a metal-binding site in FrmR. However, titration of FrmRE64H or FrmR with Co(II) results in the appearance of spectral features in the region of 330 nm, indicative of S → Co(II) ligand-to-metal charge transfer (LMCT) bands consistent with Co(II) binding to both proteins (Fig. 3A and E). For FrmR and FrmRE64H, the intensities of the feature at saturation ~0.9 × 10⁶ M⁻¹ cm⁻¹, are consistent with a single thiolate ligand (50). The intensities of a second set of Co(II)-dependent features in the region of 600 nm, indicative of d-d transitions (50), suggest tetrahedral coordination geometry. Binding curves are linear up to one equivalent of Co(II) implying K₉₅R, too tight to estimate by this method (Fig. 3A and E, insets). Cu(I)-dependent features similarly indicate tight binding of at least one equivalent of metal, and one equivalent of Cu(I) binds sufficiently tightly to co-migrate with either protein during size exclusion chromatography (Fig. 3B, C, F, G). One equivalent of Zn(II) (which is spectrally silent) also co-migrates with each protein during size exclusion chromatography (Fig. 3D and H). Preliminary Ni(II)-binding experiments with FrmRE64H were ambiguous but because no in vivo nickel-response had been detected for FrmRE64H, Ni(II)-affinities were not pursued.
Generation of a metal-sensor

12 M) and FrmRE64H (42.7 μM, monomer) or FrmR (39.9 μM, monomer), respectively (Fig. 4C and D). Again, data were fit to models describing binding of three molar equivalents of Zn(II) per tetramer (as expected, the fourth sites did not show competition with quin-2) with dashed lines describing simulated curves for \( K_{Zn1-3} \) ten-fold tighter or ten-fold weaker than the calculated affinity of the proteins. Mean values of \( K_{Zn1-3} = 2.33 (±0.3) \times 10^{-11} \) M and \( 1.7 (±0.7) \times 10^{-10} \) M for FrmRE64H and FrmR, respectively, are thus within the range of this assay (Fig. 4C and D, Table 1).

Co(II) affinities of FrmRE64H (49.3 μM, monomer) and FrmR (41.9 μM, monomer) were first analysed by competition with 10.3 μM and 9.8 μM fura-2, respectively (\( K_{Co1} \) fura-2 = 8.64 × 10⁻⁹ M) (Fig. 4E and F) (6, 47). Only FrmRE64H showed competition with fura-2 (Fig. 4E). The data were fit to a model describing binding of one Co(II) ion per FrmRE64H tetramer, which significantly departs from simulated curves describing \( K_{Co1} \) ten-fold tighter (Fig. 4E); with \( K_{Co1} \) from triplicate assays = 2.56 (±0.4) × 10⁻⁷ M (Table 1). In contrast, both FrmRE64H (87.0 μM, monomer) and FrmR (83.9 μM, monomer) showed competition with a large excess (50 mM) of Bis-Tris (\( K_{Co1} \)) Bis-Tris = 2.26 × 10⁻² M) (Fig. 4G and H) (48, 53). The data were fit to a model describing binding of four Co(II) ions, with equal affinity, per tetramer which significantly departs from simulated curves describing \( K_{Co1-4} \) ten-fold weaker. For FrmR the curves also depart from simulated curves describing \( K_{Co1-4} \) ten-fold tighter. \( K_{Co1-4} \) from triplicate assays = 7.59 (±0.4) × 10⁻⁶ M for FrmR, whereas only a weaker limit (< 10⁻⁶ M) for FrmRE64H \( K_{Co1-4} \) could be determined (Table 1).

Cuprous affinities of both proteins were determined using bicinechonic acid (BCA; \( \beta_2 = 10^{17.2} \) M⁻²) revealing competition in each case for 2 molar equivalents of Cu(I) per monomer, but with greater competition and hence tighter affinity for FrmRE64H than FrmR (Table 1, Fig. 4I and J). The data were fit to models describing binding of eight Cu(I) ions per tetramer (see Table 1 footnotes for details) which for FrmR depart from simulated curves describing binding of the tightest two Cu(I) ions (\( K_{Cu1-2} \)) ten-fold tighter and ten-fold weaker than the fitted value (Fig 4I), giving FrmR \( K_{Cu1-2} = 4.9 (±1.6) \times 10^{-15} \) M (Table 1). In contrast, \( K_{Cu1-2} \) for FrmRE64H is too tight to measure by this assay. However FrmRE64H does not significantly compete with 10 μM BCS (\( \beta_2 = 10^{19.8} \) M⁻²) (48), with saturation of the BCS₂Cu(I) complex observed at ~ 5 μM CuCl (Fig. 4K). These data imply that FrmRE64H \( K_{Cu1-2} \) can only marginally depart from the value estimated using BCA (\( K_{Cu1-2} ~ 5 \times 10^{-16} \) M) (Table 1). It is noted that the final absorbance for the BCS₂Cu(I) complex in the presence of protein was lower than predicted from its known extinction coefficient, hence the possibility of a ternary complex cannot be ruled out. In summary, the two metals which FrmRE64H now detects, Co(II) and Zn(II), bind approximately an order of magnitude more tightly than to FrmR (Table 1).

Cognate \( K_{metal} \) of Salmonella Zn(II), cobalt and Cu(I)-sensors ZntR, Zur, RcnR and CueR- If metal-sensing is dictated by relative affinity within the set of Salmonella metal-sensors, the affinity of FrmRE64H for Zn(II) and Co(II) would need to become comparable to cellular sensors for these metals. Conversely, Cu(I) affinity would need to remain weaker than Cu(I)-sensing CueR making Cu(I) still undetectable (40, 41, 54). The Salmonella sensors for Zn(II) and Co(II) are confirmed here as ZntR, Zur and RcnR (Fig. 5) (55, 56). Expression is induced from \( P_{zntA} \) and \( P_{rcnA} \) in wild type cells exposed to MNIC ZnSO₄ and CoCl₂, respectively (Fig. 5A and B). Notably minimal media for this strain (SL1344) requires histidine which may influence Ni(II) availability. Titration of ZntR with Co(II), as a spectral probe for Zn(II) binding sites, generated features diagnostic for LMCTs and d-d transitions consistent with ~ three thiolate-Co(II) bonds per ZntR monomer and tetrahedral co-ordination geometry (Fig. 5C) (50). These features saturate at ~ one equivalent of Co(II) and are bleached by addition of ~ one equivalent of Zn(II) (Fig. 5D). Zn(II) (~ one equivalent) also quenched ZntR auto-fluorescence (Fig. 5E). Salmonella ZntR is expected to be a dimer based on similarity to the E. coli homologue (49), implying a stoichiometry of two Zn(II) ions per dimer. Titrations of 18.6 μM quin-2 and ZntR (16.0 μM, monomer) with Zn(II) were fit to models describing detectable binding of two distinguishable Zn(II) ions per dimer (\( K_{Zn1} \) and \( K_{Zn2} \)); estimated mean values are shown in Table 1 (Fig. 6A). The optimised curves depart from simulated curves describing \( K_{Zn1} \) or \( K_{Zn2} \) ten-fold tighter or ten-fold weaker than their fitted values, although \( K_{Zn2} \) does approach the simulated curve describing \( K_{Zn2} \) ten-fold weaker.
but approaches a simulated curve describing simulated curve describing dimer, but not site 4) with mean values for Zn(II) per monomer (exchangeable sites 1-3 per were titrated with Zn(II) and fit to models weaker than the fitted value. ten-fold weaker (Fig. 6B). Zur-family members exist as co-ordinating thiol groups, which saturate accessible to both Co(II) and Zn(II). A total of 35.5 µM Zn(II) is required to fully saturate Zur (11.7 µM, monomer) in the presence of excess (1 mM) EDTA (Fig. 5F). In the absence of EDTA at least one further equivalent of Zn(II) binds sufficiently tightly to co-migrate with the protein during size exclusion chromatography (Fig. 5F). Titration of apo-Zur (Zn(II)-saturated at the structural site) with Co(II) generated features diagnostic for LMCTs and d-d transitions consistent with two to three co-ordinating thiol groups, which saturate between 1.5 to 2 equivalents of Co(II) per monomer (Fig. 5G) (50). These features are bleached with addition of 1.5 to 2 equivalents of Zn(II) (Fig. 5H). Zur-family members exist as dimers (57-59), and here data shows there are at least three exchangeable sites per dimer which are accessible to both Co(II) and Zn(II). A total of 35.5 µM Zn(II) is required to fully saturate Zur (11.7 µM, monomer) and mag fura-2 (12.1 µM), consistent with two monomer equivalents ((2 × 11.7 µM) + 12.1 µM = 35.5 µM) of exchangeable Zn(II) binding to Zur (:. four sites per dimer) with sufficient affinity to show some competition with mag fura-2. Of these, an estimated three sites per dimer completely withhold Zn(II) from mag fura-2 (Fig. 5I). The data in Fig. 5I were fit to a model describing four exchangeable sites per Zur dimer with dashed lines representing simulated curves describing KZn1 ten-fold tighter and ten-fold weaker than the fitted KZn4 value, and a tighter limit for KZn4 was estimated from replicate titrations (Table 1). To estimate KZn1-2 and KZn3, quin-2 (9.6 µM) and Zur (13.7 µM, monomer) were titrated with Zn(II) and fit to models describing competition from 1.5 equivalents of Zn(II) per monomer (exchangeable sites 1-3 per dimer, but not site 4) with mean values for KZn1-2 and KZn3 shown in Table 1 (Fig. 6B). The optimised curve departs from simulated curves describing KZn1-2 ten-fold tighter or ten-fold weaker than the fitted value. KZn3 departs from a simulated curve describing KZn3 ten-fold tighter, but approaches a simulated curve describing KZn3 ten-fold weaker (Fig. 6B).

Generation of a metal-sensor

Titration of RcnR with Ni(II) or Co(II) generated spectral features which saturated at one equivalent of metal (Fig. 5J and K). Ni(II)-RcnR demonstrated features < 300 nm and weak d-d transitions consistent with a six co-ordinate octahedral Ni(II)-binding site, as seen for E. coli RcnR (17). An additional Co(II)-dependant feature at 314 nm appeared with time (Fig. 5L.). Co(II)- dependent fluorescence quenching of fura-2 (13.2 µM) in the presence of RcnR (18.4 µM, monomer) was fit to a model describing competition from three sites per RcnR tetramer with two sites (KCo1-2) tighter than the third (KCo3) (Fig. 6C). The optimised curve departs from simulated curves describing KCo1-2 ten-fold tighter or ten-fold weaker, and KCo3 ten-fold tighter, than the respective fitted values. Mean values (generated from multiple titrations) for KCo1-2 and a range for KCo3 are shown in Table 1.

Salmonella CueR out-competes a ten-fold molar excess of BCS (41), and here a 100-fold and then a 75-fold excess of BCS (the latter in Fig. 6D), was used to estimate KCu1 (Table 1). In summary, the tightest exchangeable sites of the endogenous metal-sensors are tighter for their cognate metals than either FrmR or FrmRE64H, in every case (Table 1). However, the difference in KZn3 between FrmRE64H and cognate Zn(II) sensors is the smallest.

Cognate metal sensors out-compete FrmR for metal- To confirm, or otherwise, that FrmR Kmetal is weaker than CueR KCu1, ZntR KZnt1-3, and RcnR KCo1-3, pair-wise competitions were conducted for the tightest metal-binding site in which metallated FrmR was incubated with apo-forms of the respective sensors. Cu(I)-FrmR co-migrates with copper following heparin affinity chromatography (Fig. 7A). However, after mixing Cu(I)-FrmR with apo-CueR (which can be differentially resolved), copper migrates with CueR (Fig. 7A). Likewise after mixing Zn(II)-FrmR with apo-ZntR, zinc predominantly migrates (using different fractionation buffers to those in Fig. 7A) with ZntR (> 90% of control) (Fig. 7B). Diagnostic spectral features (d-d transitions) which discern Co(II)-FrmR, with tetrahedral binding geometry, from Co(II)-RcnR, with octahedral binding geometry, are lost upon addition of apo-RcnR to Co(II)-FrmR (Fig. 7C). Thus, in every case the cognate sensor out-competes FrmR confirming that FrmR Kmetal is weaker. Relative (to the cognate sensors) metal affinity could account for
why wild type FrmR does not respond to metals within cells.

Figure 8A compares the calculated fractional occupancies of the tightest exchangeable sites (from $K_{metal}$ in Table 1) of FrmR and FrmRE64H for Zn(II), Cu(I) and Co(II) with the respective cognate Salmonella sensors, as a function of metal concentration. To detect Cu(I), FrmRE64H would require intracellular buffered Cu(I) concentrations to rise ~ three orders of magnitude higher than necessary for detection by CueR, which could explain why FrmRE64H remains unresponsive to Cu(I). In contrast, partial Zn(II) occupancy of FrmRE64H will occur at Zn(II) concentrations below those required to saturate ZntR (Fig. 8A). Thus, theoretically, a ten-fold increase in $K_{Zn(II)}$ of FrmRE64H relative to FrmR may be sufficient to enable some Zn(II)-detection within the cell.

Glutathione enhances metal-detection by FrmRE64H and RcnR- In addition to responding to Zn(II), the FrmRE64H variant also responds to cellular cobalt (Fig. 2D), yet $K_{Co(II)}$ for FrmRE64H is ~ 500-fold weaker than the endogenous cobalt sensor RcnR (Fig. 8A, Table 1). An ~ ten-fold increase in $K_{Co(II)}$ alone cannot readily explain why this variant of FrmR has become responsive to cobalt. Recent studies of the complement of metal sensors from a cyanobacterium concluded that the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18). In that system, a substantial kinetic component was invoked for the detection of Zn(II) and nickel matched predictions based upon equilibrium thermodynamics, but this was untrue for cobalt (6, 11, 18).

The possibility that glutathione is required for the detection of cobalt (and Zn(II)) by FrmRE64H was investigated in $\Delta$frmR/$\Delta$gshA cells containing P$_{frmRA}$-frmRE64H fused to lacZ (Fig. 8B and C). Cells lacking glutathione showed a negligible response to either metal. Previous studies of Zn(II)-sensors have found that the low molecular weight thiol, bacillithiol, competes for metal thus reducing responses (61). ZntR-mediated expression in response to Zn(II) from the zntA promoter shows negligible difference in $\Delta$gshA cells compared to wild type (Fig. 8D). However, in common with regulation by FrmRE64H, the response of RcnR to cobalt was also reduced, but not lost, in cells missing glutathione (Fig. 8E). Thus, glutathione aids the detection of cobalt by two different sensors but has varied effects on Zn(II)-sensing.

Basal repression by FrmRE64H is less than by FrmR- The tightening of $K_{Zn(II)}$ (and $K_{Co(II)}$) is modest suggesting that additional factors might contribute to the gain of metal-detection by FrmRE64H (Fig. 2D and A, Table 1). It was noted that basal expression from the frmRA promoter is greater in cells containing FrmRE64H compared to wild type FrmR (Fig. 2D and E). Expression remains elevated in cultures treated with EDTA or the Zn(II)-chelator TPEN, implying that this is not a response to basal levels of intracellular metal (Fig. 9A, and B). As a control, ZntR-mediated $\beta$-galactosidase expression from the zntA promoter does decline upon equivalent treatment with EDTA, or TPEN (Fig. 9C and D). Furthermore, because metal-responsiveness from P$_{frmRA}$-frmRE64H is affected by glutathione (Fig. 8B and C), glutathione levels were measured but found to be not significantly altered between $\Delta$frmR cells expressing P$_{frmRA}$-frmR or P$_{frmRA}$-frmRE64H in either the presence (3.8 (±0.5) and 4.5 (±0.6) mM, respectively) or absence (4.4 (±0.8) and 3.3 (±0.4) mM, respectively) of added Zn(II).

Codon optimisation or de-optimisation alters FrmRE64H or FrmR cell$^+$ but does not switch metal-perception- Loss of repression by FrmRE64H compared to FrmR could in theory be due to reduced protein abundance, for example due to impaired stability of the mutant protein. To test this suggestion, constructs were generated in which FrmRE64H codons were optimised for efficient translation (62, 63), designated P$_{frmRA}$-frmRE64H$^{UP}$. Conversely, FrmR expression was de-optimised by introduction of rare arginine codons (62, 63), designated P$_{frmRA}$-frmR$^{DOWN}$. This approach was chosen in order to alter abundance of the proteins while preserving the transcriptional architecture. Basal expression was enhanced in cells containing frmR$^{DOWN}$ and reduced in cells containing frmRE64H$^{UP}$ relative to the respective controls and yielding matched levels of basal lacZ expression by frmR$^{DOWN}$ versus frmRE64H$^{UP}$ (Fig. 9E). Moreover, the numbers of FrmRE64H and FrmR tetramers per cell, as determined by quantitative mass spectrometry were indeed increased and decreased respectively, in cells harbouring the codon altered variants (Fig. 9G-K). Cells containing any of the variants, frmRE64H,
Production of metal-sensor FrmRE64H

Elevated basal expression of FrmRE64H, in contrast to FrmR, was unexpected when FrmRE64H was subjected to metal-binding conditions (Fig. 9K). Notably, the abundance of FrmRE64H is no less than FrmR (Fig. 9K), and an alternative explanation is needed for elevated basal expression in cells containing FrmRE64H.

\[ \Delta G_{C_{\text{Zn(II)-FrmRE64H-DNA}}} \text{is less than } \Delta G_{C_{\text{Zn(II)-FrmR-DNA}}} \]

with apo-FrmRE64H. K_{DNA} being weaker-

Fluorescence anisotropy was used to monitor interactions between either FrmRE64H or FrmR and a fluorescently labelled double stranded DNA fragment of the target operator-promoter, frmRPA pro (Fig. 1C). DNA-protein stoichiometry was first determined by monitoring DNA-binding to a relatively high concentration of frmRPA pro (2.5 µM) with saturation observed at ~ 20 µM FrmRE64H or FrmR (monomer) consistent with binding of two tetramers (Fig. 10A and B). A limiting concentration of frmRPA pro (10 nM) was subsequently titrated with apo- or Zn(II)-saturated FrmRE64H or FrmR in the presence of 5 mM EDTA or 5 µM Zn(II) respectively, and anisotropy data fitted to models describing the binding of two non-dissociable protein-tetramers per DNA molecule (Fig. 10C and D). The calculated DNA binding affinities (n ≥ 3) are shown in Table 2. K_{DNA} was similarly determined for Cu(I)-FrmR (Table 2), but weak K_{Cu(II)} precluded equivalent K_{DNA} estimations for Co(II)-saturated proteins (Table 1). Metal-binding weakens DNA binding, but unexpectedly this is true of wild type FrmR as well as FrmRE64H.

The degree to which metal-binding allosterically inhibits DNA-binding has previously been expressed as the coupling free energy (\(\Delta G_C\)) calculated from the ratio of K_{DNA} of apo- and holo-proteins and using a standard thermodynamic function (9, 11, 64, footnotes to Table 2). This yields Zn(II)-FrmR \(\Delta G_C = +2.03 \pm 0.08\) kcal mol\(^{-1}\) (\(\Delta G_{C_{\text{Zn(II)-FrmR-DNA}}}\)) and Zn(II)-FrmRE64H \(\Delta G_C = +1.24 \pm 0.16\) kcal mol\(^{-1}\) (\(\Delta G_{C_{\text{Zn(II)-FrmRE64H-DNA}}}\)) (Table 2). Unexpectedly, this approach revealed that Zn(II) is less, not more, allosterically effective when binding to FrmRE64H than to FrmR, with the former having the smaller coupling free energy. However, inspection of the DNA-binding curves (Fig. 10C and D), and K_{DNA} values (Table 2), reveals that this results from apo-FrmRE64H having a weaker DNA affinity than apo-FrmR. These data explain the loss of basal repression by FrmRE64H. But importantly, despite a lesser \(\Delta G_C\), because K_{DNA} of Zn(II)-FrmRE64H is not tighter than Zn(II)-FrmR (Table 2), at equivalent Zn(II)-saturation DNA occupancy by FrmRE64H will still be less than FrmR, in effect rendering FrmRE64H more sensitive to de-repression. Moreover, assuming a closed system, coupled thermodynamic equilibria infer that any effect of metal-binding on K_{DNA} is reciprocated in an effect of DNA binding on K_{Zn(II)} (Fig. 10E) (9, 61, 65, 66). Thus a smaller \(\Delta G_C\) means an even tighter K_{Zn(II)} for DNA-bound FrmRE64H relative to FrmR. The inferred K_{Zn(II)} sensor-DNA (on-DNA) is 5.3 \times 10^{-9} and 1.9 \times 10^{-10} M for FrmR and FrmRE64H, respectively. A weaker K_{DNA} thereby contributes in two ways to the mechanism enabling metal-perception by the FrmRE64H variant, and overall, a tighter K_{Zn(II)} plus a weaker K_{DNA} act in combination to confer Zn(II)-sensing.

**DISCUSSION**

Substitution of one amino acid has created a metal-sensor from the formaldehyde-responsive, DNA-binding, transcriptional de-repressor, FrmR (Fig. 2). Contrasting the biochemical properties of these two proteins (FrmR and FrmRE64H) along with endogenous *Salmonella* metal-sensors (Fig. 3-7, 8A, 10A and B, Tables 1 and 2), demonstrates what is required for metal-sensing within cells. These data test (by gain-of-function) theories which have been developed from correlations between the biochemical properties of various metal-sensor proteins and the metals they detect (1, 2, 6, 9, 11, 18). The single residue change in FrmRE64H tightens K_{Zn(II)} by ~ ten-fold and weakens apo-K_{DNA} by ~ ten-fold, and in combination these changes to metal-binding and DNA-binding make Zn(II)-sensitivity comparable to endogenous Zn(II)-sensors ZntR and Zur (Fig 2, 8A, 10A and B). In common with recent studies of cobalt-detection in other cells (6, 67), relative access (a major kinetic contribution) is invoked to explain the gain of cobalt detection by FrmRE64H, a response which is assisted by glutathione (Fig. 8).

Unusually, the native FrmR protein binds Co(II), Zn(II) and Cu(I) (Fig. 3 and 4). Moreover, Zn(II) and Cu(I) are shown by fluorescence anisotropy to be allosterically effective and able to weaken K_{DNA}, thereby raising questions about why native FrmR does not normally de-repress gene expression in response to...
these metal ions (Fig. 10C, and Table 2). Crucially, by characterising *Salmonella* ZnR and RcnR (Fig 5A-E and 5J-L), and by measuring cognate $K_{\text{metal}}$ of *Salmonella* Zn(II)-sensing ZnR and Zur, Cu(I)-sensing CueR and cobalt-sensing RcnR (Fig. 5F-I and 6, Table 1), it becomes evident that in each case the respective metal-affinity of FrmR is substantially weaker than each cognate sensor and it cannot compete (Fig. 7 and 8A). Values for $K_{\text{Zn(II)}}$, $K_{\text{Co(II)}}$ and $K_{\text{Cu(I)}}$ for *Salmonella* Zur, ZntR, RcnR and CueR determined here, are comparable to analogous sensors from some other organisms (1, 11, 17, 28, 64, 68) (Fig. 5 and 6). The ability of FrmR to respond to metals in vitro but not within cells (Fig. 2A and E, 10C), coupled with relative $K_{\text{metal}}$ Values (Table 1), provides another line of evidence that metal-sensing within cells is a combined product of a set of sensors (1). The best sensor in the set is the one that responds to each element (1, 11). In each case $K_{\text{metal}}$ for FrmR is substantially weaker than the respective $K_{\text{metal}}$ for the best in the set of sensors in *Salmonella* (Fig. 8A), and so it does not respond.

The E64H substitution was intended to create a metal-binding site more analogous to RcnR and indeed $K_{\text{Co(II)}}$ plus $K_{\text{Zn(II)}}$ and $K_{\text{Cu(I)}}$ are all tighter by ~ ten-fold compared to FrmR (Fig. 4 and Table 1), but all remain weaker than the respective cognate metal-sensor (Table 1, Fig. 6 and 8A). Nonetheless for Zn(II), the affinity of FrmRE64H approaches that of known Zn(II)-sensors such that there is overlap in fractional metal occupancy curves as a function of [Zn(II)] (Fig. 8A). The free energy coupling Zn(II)-binding to DNA-binding for FrmRE64H also changes relative to FrmR (Fig. 10C and D, Table 2). However, the change is the opposite of what might be predicted (1, 9, 11, 64), with Zn(II) appearing to be less, not more, allosterically effective in the mutant protein ($\Delta G^R_{\text{C}}$ Zn(II)-FrmRE64H·DNA $< \Delta G^R_{\text{C}}$ Zn(II)-FrmR·DNA). Importantly, these values incorporate a much weaker $K_{\text{DNA}}$ for apo-FrmRE64H (Fig. 10D), which lowers overall promoter occupancy enhancing sensitivity to de-repression. Moreover, if regulation is dominated by metal-binding to the DNA-protein complex to promote DNA-dissociation, then the lesser $\Delta G_C$ of FrmRE64H infers an even tighter $K_{\text{Zn(II)}}$ (assuming a closed system (9, 61, 65, 66)) of the active DNA-bound species relative to FrmR (Fig. 10E).

Unlike for Zn(II), the enhanced $K_{\text{metal}}$ of FrmRE64H does not approach that of cognate sensors for Cu(I) or cobalt (Fig. 8A). Thus relative affinity is consistent with the continued inability of FrmRE64H to detect Cu(I). However, the gain of cobalt-sensing by FrmRE64H is enigmatic. In studies of the model cyanobacterium, *Synechocystis* PCC 6803, the detection of nickel and Zn(II) correlated with relative affinity and relative-allostery within the set of sensors, but the detection of cobalt was attributed to relative access (1, 6, 11, 18). Somehow, the cobalt effector was preferentially available to the cobalt-sensor CoaR relative to sensors for other metals. Thus, although Zn(II)-sensors ZiaR and Zur had tighter affinities for Co(II) than CoaR and both were (allosterically) responsive to Co(II) in vitro, neither ZiaR nor Zur responded to cobalt in the cell, while CoaR with weaker $K_{\text{Co(II)}}$ responded (6). Unlike *Synechocystis* CoaR, since FrmR has not evolved to detect cobalt, it is difficult to understand why cobalt should be channelled to FrmRE64H (Fig. 8A, Table 1). FrmR and cobalt-sensing RcnR do share common ancestry and so interaction with a cobalt donor could perhaps be an evolutionary relic. Glutathione complexes are components of the buffered cellular pools for a number of metals (69). Since the substrates for formaldehyde dehydrogenase, FrmA, which is regulated by FrmR, are S-(hydroxymethyl)glutathione and S-nitroso glutathione, it is also formally possible that FrmR can respond to glutathione adducts (33, 70). Here we see that cobalt- and Zn(II)-sensing by FrmRE64H is somehow assisted by glutathione (Fig. 8B and C). This is opposite to what has previously been observed in the detection of cellular Zn(II) in other systems where the glutathione-substitute, bacillithiol, competes with Zn(II)-sensors (61), and here we see negligible effect of glutathione on Zn(II)-sensing by ZntR (Fig. 8D). Whether glutathione aids the detection of cobalt due to cobalt-binding and -trafficking, or due to redox effects on the oxidation state of cobalt or its ligands, remains to be established.

Basal repression by FrmRE64H is less than FrmR and this is explained by weaker $K_{\text{DNA}}$ of apo-FrmRE64H (Fig. 9, 10C and D). In pursuing the explanation for this phenotype, the abundance of both proteins was adjusted by optimising or de-optimising codons, an approach which preserves the transcriptional architecture. These changes were confirmed to increase and decrease the number of copies of FrmRE64H and FrmR per cell respectively, with concomitant gain and loss of repression leading to matched levels of
basal expression (Fig. 9E-K). However, the magnitude of these changes in protein abundance alone was insufficient to switch FrmR into a metal-sensor or to stop FrmRE64H from responding to Zn(II) or cobalt (Fig. 9F). Nonetheless, in theory, a change in relative protein abundance could alter metal competition with other sensors by mass-action, and relative abundance should be added to the list of relative properties (affinity, allostery, access) that determine which sensor is the best in the set to respond to a metal.

By how much do tighter $K_{Zn(II)}^{DNA}$ and weaker $K_{DNA}$ of the apo-protein, enhance the sensitivity of FrmRE64H to Zn(II), alone and in combination? By using the parameters set out in Tables 1 and 2, plus Fig. 9K, it has become possible to estimate fractional occupancy of the frmRA operator-promoter with repressor, either FrmR or FrmRE64H, as a function of [Zn(II)] (refer to Experimental Procedures, supplemental data, Fig. 10F-I). First, a weaker $K_{DNA}$ of apo-FrmRE64H causes operator-promoter occupancy to be less than FrmR even in the absence of elevated Zn(II) (Fig. 10F), which explains the small but detectable (Fig. 2 and 9), basal de-repression. Individually, which explains the small but detectable (Fig. 10F), which explains the small but detectable (Fig. 2 and 9), basal de-repression. Individually, the determined tighter $K_{Zn(II)}$ or weaker apo-$K_{DNA}$ alone enhance the sensitivity of FrmRE64H to [Zn(II)] by ~ one order of magnitude (dotted and dashed lines on Fig. 10F), while in combination they increase sensitivity by ~ two orders of magnitude. If regulation is dominated by metal-binding to (and promoting dissociation of) DNA-bound protein, then the inferred (weaker) $K_{Zn(II)}$ of the DNA-adduct becomes the relevant parameter (Fig. 10E). Under this regime (which assumes a closed system) the weaker $K_{DNA}$ of apo-FrmRE64H lessens $A_{Zn(II)-FrmRE64H·DNA}$ and infers a tighter $K_{Zn(II)}$ of DNA-bound FrmRE64H (on-DNA) relative to FrmR: This, in combination with the measured tighter $K_{Zn(II)}$, enhances sensitivity to [Zn(II)] by ~ three orders of magnitude (Fig. 10H).

There is ambiguity about the buffered concentrations of metals in cells (1). These values are important because relative metal-availability influences metal occupancy by metalloproteins (71, 72). Plausible limits on cellular buffered [Zn(II)] are defined by FrmRE64H, FrmR, FrmRE64H$^{UP}$ and FrmR$^{DOWN}$ (Fig. 10F to I). In the absence of elevated exogenous Zn(II), for FrmRE64H to fully repress, the buffered [Zn(II)] must be held below $10^{-11}$ M, even if the inferred weaker (on-DNA) $K_{Zn(II)}$ is applied to all molecules (Fig. 10H). This low (sub nM) value suggests that metalloproteins acquire competitive metals such as Zn(II) when there is no hydrated metal-pool. However, the estimates of the buffered concentration of Zn(II) are consistent with a hypothesis that metalloproteins acquire Zn(II) via associative ligand exchange from a polydisperse buffer (1), rather than a hydrated pool of ions: This represents an associative cell biology of Zn(II).

Whether or not a significant pool of hydrated ions contributes to the metallation and hence regulation of FrmRE64H (and by inference other metal-sensors) remains unresolved (73). One view is that metal-sensors respond to hydrated ions at ~ $10^{-8}$ M once the buffer is saturated (73). For FrmR$^{DOWN}$ to be unresponsive when cells are challenged with elevated exogenous Zn(II), the buffered [Zn(II)] must remain somewhere below $10^{-8}$ M, even if the inferred weaker (on-DNA) value for $K_{Zn(II)}$ is assigned to all FrmR molecules (Fig. 10I). This limit drops to $10^{-10}$ M, if the determined (off-DNA) $K_{Zn(II)}$ is used (Fig. 10G). Conversely, for FrmRE64H to respond, the buffered [Zn(II)] need only exceed $10^{-11}$ M using the inferred weaker (on-DNA) $K_{Zn(II)}$ (Fig. 10H). This places the intracellular [Zn(II)] at which FrmRE64H responds to somewhere within the range $10^{-11}$ to $10^{-8}$ M.

A long-term aspiration is to gather analogous $K_{DNA}$ values, for cognate and non-cognate metals, plus protein abundance, for a cell’s complement of metal-sensors. In this manner, comparative models of sensor occupancy with metal (as in Fig 8A), could be refined to more sophisticated comparative models of promoter occupancy by repressors, as shown here in Figure 10 panels F to I. In turn, this should render transcriptional responses to metals predictable. In closing, the (subtle) biochemical changes which in combination enable FrmRE64H to detect a sub-set of metals, supports a view that (modest) differences in the relative properties of a cells’ complement of sensors dictate which sensor is the best in the set to detect each metal inside cells.
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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

DO and CP made equivalent contributions to the conduct of the in vitro experiments, analysis and preparation of the data and are joint first authors. DO did the in vivo experiments. NJR and DO drafted the manuscript, interpreted the significance of the data and were responsible for the iterative design of experiments. JC and TGH performed the quantitative LC-MS/MS. AWF, BC and DO developed the fractional occupancy models. NJR and EL-L were responsible for the conception of the programme. All authors reviewed the results, edited and approved the final version of the manuscript. NJR coordinated and designed the study, and provided intellectual input into all aspects of the research.

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**FOOTNOTES**

The abbreviations used are: TMAO, trimethylamine-N-oxide; MNIC, maximum non-inhibitory concentrations; IPTG, 1-thio-β-d-galactopyranoside; TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine; ICP-MS, inductively coupled plasma-mass spectrometry; LMCT, ligand-to-metal charge transfer; BCS, bathocuproine sulfonate; BCA, bicinechinonic acid.
FIGURE LEGENDS

FIGURE 1. FrmR candidate metal binding ligands and genetic organisation. A, Dimeric representation of Mycobacterium tuberculosis Cfr (Cfr_Mtb), Synechocystis PCC 6803 InrS (InrS SYN), Salmonella RcnR (RcnR_STY), E. coli RcnR (RcnR_Ec), Salmonella FrmR (FrmR_STy) and E. coli FrmR (FrmR_Ec). The residues at positions of the WXYZ fingerprint are highlighted (15, 17). B, Alignment of M. tuberculosis CsoR (CsoR_Mtb), Synechocystis PCC 6803 InrS (InrS_SYN), Salmonella RcnR (RcnR_STY), E. coli RcnR (RcnR_Ec), Salmonella FrmR (FrmR_STy) and E. coli FrmR (FrmR_Ec). The residues at positions of the WXYZ fingerprint are highlighted (15, 17).

FIGURE 2. A single residue change renders Salmonella FrmR responsive to cobalt and Zn(II), in addition to formaldehyde. A, β-galactosidase activity in ΔfrmR containing P\textsubscript{frmRA}ΔfrmR fused to lacZ grown to mid-exponential phase in M9 minimal medium in the absence or presence of MNIC MnCl\textsubscript{2}, CuCl\textsubscript{2}, NiSO\textsubscript{4}, CuSO\textsubscript{4}, ZnSO\textsubscript{4}, or formaldehyde (HCHO). B, Expression from P\textsubscript{frmRA}ΔfrmR, P\textsubscript{frmRA}ΔfrmR, following growth to mid-exponential phase in the absence or presence of MNIC formaldehyde. D, Expression from P\textsubscript{frmRA}ΔfrmRE64H, or E, P\textsubscript{frmRA}ΔfrmR as a function of time following exposure of logarithmic cells to MNIC Mn(II) (open circles), Fe(III) (filled squares), Co(II) (open squares), Ni(II) (filled diamonds), Cu(II) (open diamonds), Zn(II) (filled triangles), formaldehyde (open triangles) or untreated control (filled circles).

FIGURE 3. FrmRE64H and FrmR bind Co(II), Cu(I) and Zn(II). Apo-subtracted UV-visible difference spectra of A, FrmRE64H (87.0 µM, monomer) upon titration with CoCl\textsubscript{2} and binding isotherms (inset) at 336 nm (triangles) and 614 nm (squares) and B, FrmRE64H (21.5 µM, monomer) upon titration with CuCl and binding isotherms (inset) at 240 nm. C, Analysis of fractions (0.5 ml) for protein by Bradford assay (open circles) and metal by ICP-MS (filled circles) following size exclusion chromatography of FrmRE64H (0.5 ml at 50 µM, monomer) pre-incubated with 150 µM CuCl. D, As ‘C’ except with 38 µM FrmRE64H and 150 µM ZnCl\textsubscript{2}. E-H, as described for A-D but using FrmR; 83.9 µM (E); 21.3 µM (F), 50 µM (G) and 38 µM (H).

FIGURE 4. Zn(II), Co(II) and Cu(I) affinities of FrmRE64H and FrmR. A, Representative (n = 3) mag fura-2 absorbance upon titration of mag fura-2 (10.1 µM) with ZnCl\textsubscript{2} in the presence of FrmRE64H (18.8 µM, monomer). B, As ‘A’ but with mag fura-2 (12.2 µM) and FrmR (20.4 µM, monomer). C, Representative (n = 3) quin-2 absorbance upon titration of quin-2 (14.1 µM) with ZnCl\textsubscript{2} in the presence of FrmRE64H (42.7 µM, monomer). D, As ‘C’ but with quin-2 (14.1 µM) and FrmR (39.9 µM, monomer). In each case (A-D), solid lines are fits to a model describing protein-competition with mag fura-2 or quin-2 for 0.75 equivalents of Zn(II) per monomer (three sites per tetramer, Kzz\textsubscript{1,3}). Dashed lines are simulated curves with Kzz\textsubscript{1,3} 10-fold tighter and 10-fold weaker. E, Representative (n = 3) fura-2 fluorescence emission (λ\textsubscript{ex} = 360 nm) upon titration of fura-2 (9.8 µM) with CoCl\textsubscript{2} in the presence (filled circles) of FrmRE64H (49.3 µM, monomer). Solid line is a fit to a model describing protein-competition for 0.25 equivalents of Co(II) per monomer (one site per tetramer, Kco\textsubscript{1}). Dashed lines are simulated curves describing Kco\textsubscript{1} 10-fold tighter and 10-fold weaker. F, Fluorescence emission of fura-2 (10.3 µM) upon titration with Co(II) as described in ‘E’ in the absence (open circles) or presence (filled circles) of FrmR (41.9 µM, monomer). G, Representative (n = 3) Co(II)-dependent absorbance at 336 nm of FrmRE64H (87.0 µM, monomer) upon titration with CoCl\textsubscript{2} in the presence of 50 mM Bis-Tris. H, As ‘G’ but with FrmR (83.9 µM, monomer). Solid lines (for ‘G’ and ‘H’), are fits to a model describing protein-competition for one molar equivalent of Co(II) per monomer (four sites per tetramer, Kco\textsubscript{1,4}). Dashed lines are simulated curves describing Kco\textsubscript{1,4} 10-fold tighter and 10-fold weaker. I, Representative (n = 3) BCA absorbance upon titration of BCA (40 µM) with CuCl in the presence of FrmRE64H (11 µM, monomer). J, As ‘I’ but with FrmR (10 µM, monomer). Solid lines (for ‘I’ and ‘K’) are fits to a model describing protein-competition with BCA for two equivalents of Cu(I) per monomer (eight sites per tetramer). Dashed lines are simulated curves with Kco\textsubscript{1,2} 10-fold tighter and 10-fold weaker. Solid red line (‘I’ only)
is a simulated curve describing $K_{Co1-2}$ 100-fold weaker. $K$, Representative (n=4) BCS absorbance upon titration of BCS (10 µM) with CuCl in the presence of FrmRe64H (29.7 µM, monomer).

FIGURE 5. Characterisation of Salmonella ZntR, Zur and RcnR. β-galactosidase activity in wild type Salmonella (defined earlier) containing A, P$_{lac}$A or B, rcnR-P$_{rcn}$ fused to lacZ following growth to mid-exponential phase in the absence or presence of MNIC MnCl$_2$, C$_6$H$_5$FeO$_7$, CoCl$_2$, NiSO$_4$, CuSO$_4$, or ZnSO$_4$. C, Apo-subtracted UV-visible difference spectra of ZntR (24.9 µM, monomer) upon titration with CoCl$_2$. Inset: binding isotherms at 314 nm (circles) and 650 nm (triangles). D, Apo-subtracted UV-visible difference spectra of Co(II)-ZntR (24.0 µM, monomer; equilibrated with 1 molar equivalent CoCl$_2$) (solid line), and following addition of 0.5 and 1 molar equivalent of ZnCl$_2$ (dashed lines). E, Fluorescence emission of ZntR (13.1 µM, monomer) following titration with ZnCl$_2$. F, Analysis of fractions (0.5 ml) for protein by Bradford assay (open circles) and zinc by ICP-MS (filled circles) following size exclusion chromatography of Zur (0.5 ml at 20 µM, monomer) pre-incubated with 1 mM EDTA (left-panel) or 120 µM ZnCl$_2$ (right panel). G, Apo-subtracted UV-visible difference spectra of Zur (24.8 µM, monomer) upon titration with CoCl$_2$. Inset: binding isotherms at 350 nm (open circles). H, Apo-subtracted UV-visible difference spectra of Zur (27.7 µM, monomer; equilibrated with two molar equivalents of CoCl$_2$) (solid line), and following titration with ZnCl$_2$ (dashed lines). Inset: quenching of feature at 350 nm. I, Representative (n = 3) mag fura-2 absorbance upon titration of mag fura-2 (12.1 µM) with ZnCl$_2$ in the presence of Zur (11.7 µM, monomer). Solid line describes competition from Zur for two equivalents of Zn(II) per monomer (four exchangeable sites per dimer, with three independent binding events: $K_{Zn1}$, $K_{Zn3}$ and $K_{Zn4}$). Dashed lines are simulated curves with $K_{Zn1}$ 10-fold tighter and 10-fold weaker than fitted $K_{Zn1}$ ($K_{Zn1}$ fixed to fitted values). J, Apo-subtracted UV-visible difference spectra of RcnR (30.6 µM, monomer) upon titration with NiCl$_2$. Inset: binding isotherm at 333 nm. K, As ‘J’ except with RcnR (27.3 µM, monomer) and CoCl$_2$. L, Apo-subtracted absorbance of RcnR (31.4 µM, monomer) after addition of 34.5 µM CoCl$_2$ and incubation at room temperature under anaerobic conditions in a gas-tight cuvette for 10 min (solid line) or 65 h (dashed line). Inset: time-course at 314 nm.

FIGURE 6. Cognate metal affinities of Salmonella ZntR, Zur, RcnR and CueR. A, Representative (n = 3) quin-2 absorbance upon titration of quin-2 (18.6 µM) and ZntR (16.0 µM, monomer) with ZnCl$_2$. Solid line describes competition from ZntR for Zn(II) per monomer (two independent sites per dimer; $K_{Zn1}$ and $K_{Zn2}$). Dashed lines describe $K_{Zn1}$ 10-fold tighter and 10-fold weaker than fitted $K_{Zn1}$ ($K_{Zn1}$ fixed to fitted $K_{Zn1}$). Dotted lines describe $K_{Zn2}$ 10-fold tighter and 10-fold weaker than fitted $K_{Zn2}$ ($K_{Zn2}$ fixed to fitted $K_{Zn2}$). B, Representative (n = 3) quin-2 absorbance upon titration of quin-2 (9.6 µM) and Zur (13.7 µM, monomer) with ZnCl$_2$. Solid line describes competition from Zur for 1.5 molar equivalents of Zn(II) per monomer (three sites per dimer with two independent binding events; $K_{Zn1}$, $K_{Zn3}$). Dashed lines describe $K_{Zn1}$ 10-fold tighter and 10-fold weaker than fitted $K_{Zn1}$ ($K_{Zn1}$ fixed to fitted $K_{Zn1}$). Dotted lines describe $K_{Zn2}$ 10-fold tighter and 10-fold weaker than the fitted $K_{Zn2}$ ($K_{Zn2}$ fixed to fitted $K_{Zn2}$). C, Representative (n = 3) fluorescence emission upon titration of fura-2 (13.2 µM) and RcnR (18.4 µM, monomer) with CoCl$_2$. Solid line describes competition from RcnR for 0.75 molar equivalents of Co(II) per monomer (three sites per tetramer, with two independent binding events; $K_{Co1}$ and $K_{Co3}$). Dashed lines describe $K_{Co1}$ 10-fold tighter and 10-fold weaker than the fitted $K_{Co1}$ ($K_{Co1}$ fixed to fitted $K_{Co1}$). Dotted lines describe $K_{Co1}$ 10-fold tighter and 10-fold weaker than the fitted $K_{Co3}$ ($K_{Co3}$ fixed to fitted $K_{Co3}$). D, Absorbance at 483 nm of BCS (750 µM) titrated with CuCl (filled circles) or BCS (750 µM) pre-equilibrated with 4 µM Cu(I) and incubated (60 min) with CueR (12.3 µM, monomer) (n = 3, open triangles). Absorbance values depicting complete, or no competition from CueR for Cu(I) are shown (open circles).

FIGURE 7. Salmonella metal-sensors compete with FrmR for their cognate metals. A, Heparin affinity chromatography of FrmR (40 µM, monomer) equilibrated with 10 µM CuCl (open circles) or with addition of 20 µM CueR, monomer (filled circles). B, As ‘A’ except with ZnCl$_2$ (10 µM) and addition of ZntR instead of CueR. ZntR does not bind the column and FrmR elutes in later fractions (relative to ‘A’ ) at this ionic strength. In each case, fractions (1 ml) were assayed for metal by ICP-MS.
and protein by SDS-PAGE (shown for the competition experiments). C, Apo-subtracted difference spectra following addition of 9.9 µM CoCl₂ to 41.5 µM FrmR monomer (dashed line), 42.2 µM RcnR monomer (dotted line) or FrmR followed by addition of RcnR (solid line). 

FIGURE 8. Comparative metal-affinities and the contribution of glutathione to metal-sensing. A, Fractional occupancy of FrmR and FrmRE64H with Zn(II), Cu(I), and Co(II) as a function of (buffered) metal concentration compared to cognate metal sensors from Salmonella: Zur and ZntR with Zn(II), CueR with Cu(I) and RcnR with Co(II). Fractional occupancy (θ) = [Metal]buffered/[K_{metal} + [Metal]buffered] using K_{metal} in Table 1. B, β-galactosidase activity in ΔfrmR (filled circles) or ΔfrmRΔgshA (open circles) containing P_{frmA}-frmRE64H following exposure (2 h) of logarithmic cells to CoCl₂. C, as ‘B’ but with ZnSO₄ instead of CoCl₂. D, β-galactosidase activity in Salmonella (wild type, defined earlier) (filled circles) or ΔgshA (open circles) containing P_{cnlA} grown as described in ‘C’. E, β-galactosidase activity in wild type (filled circles) or ΔgshA (open circles) containing rcnR-P_{rcnR} following growth in conditions described in ‘B’.

FIGURE 9. Basal expression from P_{frmA}-frmRE64H is higher than P_{frmA}-frmR. β-galactosidase activity in ΔfrmR containing P_{frmA}-frmR (filled circles) or P_{frmA}-frmRE64H (open circles) following growth to early-exponential phase in the presence of A, EDTA or B, TPEN. C, Expression from P_{cnlA} in wild type Salmonella, grown as described in ‘A’, or D, as described in ‘B’. E, Expression in ΔfrmR containing P_{frmA}-frmR (white bars), P_{frmA}-frmRE64H (dashed white bars), P_{frmA}-frmRE64H (grey bars) or P_{frmA}-frmRE64H (open circles). F, following exposure (2h) to Zn(II), Co(II) or formaldehyde, or untreated control. G-J, Multiple reaction monitoring, quantitative MS of cell extracts. Representative (n = 3) extracted LC-MS chromatograms of ion transitions detected in ΔfrmR containing G, P_{frmA}-frmR, H, P_{frmA}-frmRE64H, I, P_{frmA}-frmRE64H or J, P_{frmA}-frmRE64H. Transitions 451.24/716.4 and 456.24/726.4 are for analyte GQVEALER (solid lines) or labelled GQVEALER[^13C₆,^15N₄] (dashed lines). K, Abundance of FrmR and variants using quantitative data obtained in G-J.

FIGURE 10. Zn(II) weakens K_{DNA} of FrmR and FrmRE64H and its effect on DNA occupancy. Anisotropy change upon titration of a high concentration of frmRPro (2.5 µM) with A, FrmR, B, FrmRE64H, or C, a limiting-concentration of frmRAPro (10 nM) with apo-FrmR in the presence of 5 mM EDTA (closed symbols) or Zn(II) in the presence of 5 µM ZnCl₂ (open symbols). D, As ‘C’ but using FrmRE64H. Symbol shapes represent individual experiments. Data were fit to a model describing a 2:1 protein tetramer (non-dissociable):DNA stoichiometry, (binding with equal affinity) and lines represent simulated curves produced from the average K_{DNA} determined across the experimental replicas shown. E, The coupled thermodynamic equilibria (assuming a closed system) describing the relationship between FrmR tetramer (P), Zn(II) (Z), and P_{frmA} (D) (9, 65, 66). The coupling constant (K_{C}) is determined from the ratio K_{C} = (K_{DNA}_{Zn(II)-FrmR}/K_{DNA}_{FrmR}) (equation 1) and used to calculate K_{C} the (Zn(II) affinity of the DNA-bound protein, K_{Zn(II)}_{FrmR-DNA}) from K_{C} (K_{Zn(II)}_{FrmR}) (equation 2). F, Calculated fractional occupancy of P_{frmA} with FrmR (filled circles) and FrmRE64H (open circles) as a function of (buffered) [Zn(II)] which incorporates the determined FrmR or FrmRE64H abundance, K_{Zn(II)}_{sensor} (off-DNA) and K_{DNA} (Table 1). Additional lines represent hypothetical fractional occupancy of P_{frmA} with FrmRE64H but substituting K_{Zn(II)} (dotted) or K_{DNA} (dashed) for that of FrmR. G, As ‘F’ but using the determined abundance for FrmR (solid symbols) and FrmRE64H (open symbols). H and I, as ‘F’ and ‘G’, respectively, except using K_{Zn(II)} (on-DNA) (calculated using the equations in ‘E’).
TABLE 1
Metal affinities of FrmR, FrmRE64H, Zur, ZntR, RcnR and CueR

| Sensor | Metal   | $K_{metal}$ (M) |
|--------|---------|-----------------|
| FrmR   | Co(II)  | $K_{1.4} = 7.59 \pm 0.4 \times 10^{-6}$ |
|        | Zn(II)  | $K_{1.3} = 1.7 \pm 0.7 \times 10^{-10}$ |
|        | Cu(I)   | $K_{1.2} = 4.9 \pm 1.6 \times 10^{-15}$; $K_{1.4} = 1.72 \pm 0.7 \times 10^{-12}$; $K_{5.8} \geq 8 \times 10^{-11}$ |
| FrmRE64H | Co(II)  | $K_{1.4} = 2.56 \pm 0.4 \times 10^{-7}$; $K_{1.4} < 10^{-6}$ |
|        | Zn(II)  | $K_{1.3} = 2.33 \pm 0.3 \times 10^{-11}$ |
|        | Cu(I)   | $K_{1.2} \sim 5 \times 10^{-16}$; $K_{3.4} = 7.29 \pm 1.29 \times 10^{-15}$; $K_{4.6} = 5.6 \pm 2.0 \times 10^{-12}$; $K_{5.8} \geq 4 \times 10^{-10}$ |
| Zur    | Zn(II)  | $K_{1.2} = 6.36 \pm 0.41 \times 10^{-13}$; $K_{3.4} = 8.04 \pm 2.92 \times 10^{-11}$; $K_{4.6} \geq 5 \times 10^{-7}$ |
| ZntR   | Zn(II)  | $K_{1.2} = 3.2 \pm 0.73 \times 10^{-12}$; $K_{3.4} = 2.68 \pm 0.73 \times 10^{-11}$ |
| RcnR   | Co(II)  | $K_{1.2} = 5.06 \pm 0.86 \times 10^{-10}$; $3 \times 10^{-5} \geq K_{3.4} \geq 10^{-7}$ |
| CueR   | Cu(I)   | $K_{1}^{m} = 3.25 \pm 0.66 \times 10^{-19}$ |

a. Conditions: 10 mM HEPES pH 7.0, 100 mM NaCl, 400 mM KCl for FrmR, FrmRE64H, Zur, ZntR and CueR; 10 mM HEPES pH 7.0, 5% glycerol, 100 mM NaCl, 400 mM KCl for RcnR.

b. Fit to a model describing Co(II) binding with equal affinity to four sites ($K_{Co1-4}$) on an FrmR or FrmRE64H tetramer, determined by competition with Bis-Tris (n = 3). A weaker limit is defined for FrmRE64H.

c. Fit to a model describing Zn(II) binding with equal affinity to the first three sites ($K_{Zn1-3}$) on an FrmR or FrmRE64H tetramer, determined by competition with quin-2 (n = 3).

d. Fit to a model describing Cu(I) binding with equal affinity to the first two sites ($K_{Cu1-2}$), with equal affinity to sites 3 and 4 ($K_{Cu3-4}$), and with equal affinity to sites 5-8 ($K_{Cu5-8}$) on an FrmR tetramer (with $K_{Cu1-2} < K_{Cu3-4} < K_{Cu5-8}$), determined by competition with BCA (n = 4). A tighter limit is defined for FrmR $K_{Cu7-8}$.

e. Fit to a model describing Co(II) binding to the first site ($K_{Col}$) on an FrmRE64H tetramer, determined by competition with fura-2 (n = 3).

f. Fit to a model describing Cu(I) binding with equal affinity to the first two sites ($K_{Cu1-2}$), with equal affinity to sites 3 and 4 ($K_{Cu3-4}$), and with equal affinity to sites 5 and 6 ($K_{Cu5-6}$), and with equal affinity to sites 7 and 8 ($K_{Cu7-8}$) on an FrmRE64H tetramer (with $K_{Cu1-2} < K_{Cu3-4} < K_{Cu5-6} < K_{Cu7-8}$), determined by competition with BCA (n = 4). A tighter limit is defined for FrmR $K_{Cu7-8}$.

g. Approximation reflects the fact that sites 1 and 2 on an FrmRE64H tetramer outcompete BCA for Cu(I), but fail to compete with BCS (although the formation of a ternary complex cannot be ruled out).

h. Fit to a model describing Zn(II) binding to three sites ($K_{Zn1-2}$, and $K_{Zn3}$) on a Zur dimer (with the structural site already filled) with equal affinity to the first two sites ($K_{Zn1-2}$) and $K_{Zn1-2} < K_{Zn3}$, determined by competition with quin-2 (n = 3).

i. Fit to a model describing Zn(II) binding to the fourth site ($K_{Zn4}$) on a Zur dimer (with the structural site already filled), determined by competition with mag fura-2 (n = 3). Only a tighter limit can be determined.

j. Fit to a model describing Zn(II) binding to two sites ($K_{Zn1}$ and $K_{Zn2}$) on a ZntR dimer ($K_{Zn1} < K_{Zn2}$), determined by competition with quin-2 (n = 3).

k. Fit to a model describing Co(II) binding to three sites ($K_{Co1-2}$ and $K_{Co3}$) on an RcnR tetramer with equal affinity to the first two sites ($K_{Co1-2}$) and $K_{Co1-2} < K_{Co3}$, determined by competition with fura-2 (n = 3).

l. Range represents the fact that RcnR exhibits linear absorbance features upon titration with Co(II) to one molar equivalent per monomer, but site 3 does not sufficiently compete with fura-2 for Co(II).

m. Determined by competition with BCS (n = 6) and describing binding of Cu(I) to the first site ($K_{Col}$) on a CueR dimer.
TABLE 2
DNA binding affinities\textsuperscript{a,b} and allosteric coupling free energies\textsuperscript{c} of FrmR and FrmRE64H

| Sensor  | Metal | $K_{DNA}$ (M)\textsuperscript{d} | $\Delta G_c$ (kcal mol\textsuperscript{-1}) |
|---------|-------|-------------------------------|------------------------------------------|
| FrmR    | apo   | $9.94 \pm 0.3 \times 10^{-8}$  | n/a                                      |
|         | Zn(II) | $3.11 \pm 0.4 \times 10^{-6}$  | $2.03 \pm 0.08$                          |
|         | Cu(I)  | $6.54 \pm 1.3 \times 10^{-7}$  | $1.10 \pm 0.10$                          |
| FrmRE64H| apo   | $4.26 \pm 0.4 \times 10^{-7}$  | n/a                                      |
|         | Zn(II) | $3.51 \pm 0.7 \times 10^{-6}$  | $1.24 \pm 0.16$                          |

a. Determined by fluorescence anisotropy.
b. Conditions: 25 °C, 10 mM HEPES pH 7.0, 60 mM NaCl, 240 mM KCl with addition of 5 mM EDTA for apo-protein titrations or 20 µM ZnCl\textsubscript{2} for Zn(II)-protein titrations. Proteins were incubated with 1.2 molar equivalents per monomer of ZnCl\textsubscript{2} or CuCl\textsubscript{2} for metal-loaded titrations.
c. $\Delta G_c = -RT\ln K_c$
d. Fit to a model describing two non-dissociable tetramers binding to frmRAPro with equal affinity (n = 3).
Figure 1.

Generation of a metal-sensor

A

B

C

CsoR_Mtb

.......

InrS_Syn

.......

RcnR_STy

.......

RcnR_Ec

.......

FrmR_STy

.......

FrmR_Ec

.......

glutathione-dependant alcohol dehydrogenase (Zn-requiring) transcriptional regulator

TTCTGATAGTATACCCCCCTATAGTATATGGAG

FrmRA

Pro
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.

- **Panel A**: Graph showing absorption at 261 nm as a function of Zn(II) concentration. The curve is labeled with ZntR.
- **Panel B**: Graph showing absorption at 261 nm as a function of Co(II) concentration. The curve is labeled with Zur.
- **Panel C**: Graph showing intensity at 483 nm as a function of Cu(I) concentration for two conditions: Cu(I)-CueR = 0 µM and Cu(I)-CueR = 4 µM. The curves are labeled with ZntR, Zur, and RcnR.
- **Panel D**: Graph showing a linear relationship between Cu(I) concentration and absorbance at 483 nm for Cu(I)-CueR = 4 µM.
Figure 7.
Generation of a metal-sensor

Figure 8.

![Graph showing fractional sensor occupancy with metal](#)

- **A**: Fractional sensor occupancy with metal
  - X-axis: [Metal]buffered (M)
  - Y-axis: Fractional sensor occupancy with metal
  - Zn(II)
  - ZntR
  - FrmR
  - FrmRE64H
  - Zur

- **B**: β-galactosidase activity
  - X-axis: [CoCl₂] (µM)
  - Y-axis: β-galactosidase activity (nmoles o-nitrophenol min⁻¹ mg protein⁻¹ x 10⁴)

- **C**: β-galactosidase activity
  - X-axis: [ZnSO₄] (µM)
  - Y-axis: β-galactosidase activity (nmoles o-nitrophenol min⁻¹ mg protein⁻¹ x 10⁴)

- **D**: β-galactosidase activity
  - X-axis: [ZnSO₄] (µM)
  - Y-axis: β-galactosidase activity (nmoles o-nitrophenol min⁻¹ mg protein⁻¹ x 10⁴)

- **E**: β-galactosidase activity
  - X-axis: [CoCl₂] (µM)
  - Y-axis: β-galactosidase activity (nmoles o-nitrophenol min⁻¹ mg protein⁻¹ x 10⁴)
Figure 9.

| Strain                              | Tetramer cell⁻¹ |
|-------------------------------------|-----------------|
| ΔfrmR PainmentΔfrmR                | 135 ± 17        |
| ΔfrmR PainmentΔfrmRE64H             | 149 ± 4         |
| ΔfrmR PainmentΔfrmRDOWN             | 88 ± 9          |
| ΔfrmR PainmentΔfrmRE64H↑            | 176 ± 3         |
A

B

C

D

E

P + Z ⇌ K₁

P·Z

+ P·D

D

K₃

K₄

K₂

K₁

K₂ = K₃K₄

K₄

K₃

K₁

F

G

P·D + Z ⇌ (P·Z)·D

\[ K₄ = \frac{K_2}{K_3} = \frac{K_2}{K_3} \] (1)

\[ K_2 = K_1K_2 = K_3K_4 \] (2)

FramR

FramR

E64H

E64H

FramR

FramR

E64H

E64H

Fractional P FrmR occupancy

Fractional P FrmR occupancy

log[Zn(II)] (M)

log[Zn(II)] (M)
Generating a Metal-Responsive Transcriptional Regulator to Test What Confers Metal-Sensing in Cells
Deenah Osman, Cecilia Piergentili, Junjun Chen, Buddhapriya Chakrabarti, Andrew W. Foster, Elena Lurie-Luke, Thomas G. Huggins and Nigel J. Robinson

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