Fine control of metal concentrations is necessary for cells to discern zinc from cobalt

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Bacteria possess transcription factors whose DNA-binding activity is altered upon binding to specific metals, but metal binding is not specific in vitro. Here we show that tight regulation of buffered intracellular metal concentrations is a prerequisite for metal specificity of Zur, ZntR, RcnR and FrmR in Salmonella Typhimurium. In cells, at non-inhibitory elevated concentrations, Zur and ZntR, only respond to Zn(II), RcnR to cobalt and FrmR to formaldehyde. However, in vitro all these sensors bind non-cognate metals, which alters DNA binding. We model the responses of these sensors to intracellular-buffered concentrations of Co(II) and Zn(II) based upon determined abundances, metal affinities and DNA affinities of each apo- and metalated sensor. The cognate sensors are modelled to respond at the lowest concentrations of their cognate metal, explaining specificity. However, other sensors are modelled to respond at concentrations only slightly higher, and cobalt or Zn(II) shock triggers mal-responses that match these predictions. Thus, perfect metal specificity is fine-tuned to a narrow range of buffered intracellular metal concentrations.
Most bacteria contain a set of metal sensors, each responding to a specific metal ion to modulate expression of genes encoding proteins involved in metal homeostasis, which include transporters that either import specific metals during metal deficiency or export specific metals that are in excess. Correct regulation of metal homeostasis is critical for a cell to achieve metal sufficiency while avoiding metal toxicity. Metal sensors are typically allosteric transcription factors whose DNA-binding activity is altered upon metal binding, resulting in metal-dependent modification of gene expression either via co-repression (as for Zur, Fig. 1a) or de-repression (as for RcnR, Fig. 1c). A challenge exists because, in common with other proteins, metal sensors bind metal ions with an order of preference that matches the Irving–Williams series and are therefore not inherently selective for binding solely to their cognate metal (Supplementary Fig. 1).

Protein mis-metalation is a feature of metal toxicity. For example, Zn(II), cobalt (and copper) toxicity in E. coli involve mis-metalation of [4Fe-4S] clusters. Metal sensors can also be mis-metalated in vivo, e.g., both the Mn(II)-sensor MntR and Fe(II)-sensor Fur from Salmonella Typhimurium strain 14028 can respond to both Mn(II) and Fe(II) in mutants lacking the cognate metal sensor. In Bacillus subtilis, by contrast, Fe(II) is an antagonist to Mn(II) sensing by MntR, while Fur can again mal-respond to Mn(II) in mutants which overexpress Fur.

Exposure to Cd(II), Co(II) or Ni(II) dysregulates the expression of cccD, encoding a pneumococcal Zn(II)-efflux protein, suggesting mis-metalation of the Zn(II)-sensor SczA. Changes in transcription during short-term exposure to elevated metal concentrations, for example, with a copper shock, have been distinguished from those occurring after longer-term steady-state adaptation to elevated copper; the latter characterised by selective expression of known copper resistance regulons.

Protein Data Bank structures 4MTD for Zur (a), 4WLW for ZntR (b), 5LCY for both RcnR (c) and FrmR (d) with identified DNA-binding sites (bold), upstream of target genes. The DNA sequences shown were used for fluorescence anisotropy and orange bars indicate the region amplified by end point PCR and quantitative PCR. Known or inferred ligands for effector binding are enlarged. Zur contains a Cys4-structural site and metal binding are enlarged. Zur contains a Cys4-structural site and metal binding (Cys35, His60 and either His64 for FrmR/FrmRE64H, which has been exploited by immune systems.

The aim of this research was to understand how a bacterial cell selectively responds to Zn(II) and cobalt, and to discover whether metal sensing is liable to mal-respond to a wrong metal. The cognate sensors for Zn(II) (ZntR and Zur, Fig. 1a, b) and cobalt (RcnR, Fig. 1c), in the enteric pathogen Salmonella Typhimurium strain SL1344 (hereafter Salmonella) were previously identified. Products of genes regulated by ZntR and Zur are, respectively, adapted to export and import Zn(II) and not cobalt, while RcnR-regulated RcnA is adapted to export cobalt and not Zn(II), in Salmonella and/or E. coli. By analogy to E. coli, Salmonella Zur is predicted to also regulate expression of an alternate ribosomal protein that does not require Zn(II), plus a periplasmic lysozyme inhibitor. Salmonella also contains an RcnR-like sensor, FrmR (Fig. 1d), that is adapted to sense formaldehyde and does not respond during exposure to maximum non-inhibitory concentrations (MINCs) of metals, including cobalt and Zn(II). However, FrmR unexpectedly binds and allosterically responds to metals in vitro, and a single amino acid substitution generates an FrmR variant (FrmR64H), which can respond to Zn(II) and cobalt in vivo. Increased sensitivity to Zn(II) of FrmR64H is due to an ~tenfold tighter Zn(II) affinity and ~4-fold weaker DNA affinity of apo-FrmR64H, relative to FrmR. Thus, modest changes can generate a metal sensor from a non-metal sensor and this suggests that the cell may be poised close to thresholds for detecting and discerning between metals.
When the transcription of genes encoding Ni(II) import and export was engineered to rely on sensors adjusted to respond at higher Ni(II) concentrations, the cellular Ni(II) content increased relatively little and instead the sensors ceased to respond. Thus, the sensitivity of a DNA-binding metal sensor is tuned to a buffered concentration of its cognate ion, presumably to regulate mechanisms that prevent this buffer from becoming depleted or saturated with metal. Factors known to influence metal detection by each sensor are metal affinity, DNA affinity, the allosteric mechanism connecting metal binding to DNA binding, plus the abundance of sensor protein. Several of these parameters have been measured for different members of a set of sensors for Zn(II), Ni(II) and Co(II) in a common cell (Synechocystis PCC 6803). The Ni(II) sensor, InrS, has the highest affinity for Ni(II) while the Zn(II) sensor ZiaR has the greatest free energy coupling Zn(II) binding to DNA binding among the cells’ set of sensors. This illustrates how metal selectivity can be understood by comparing the relative properties of different metal sensors within a common cell. Here such observations are further rationalised by relating the sensitivities of metal sensors to buffered concentrations of the respective ions.

Ultimately, metal sensitivity of a sensor will be some function of all of the above factors operating together. However, quantitatively combining all of these factors presents a challenge. In this work, we measure these parameters and incorporate them into mathematical models in order to understand in vivo specificity of sensors to Zn(II) and Co(II). The computational methods are set out in a format to assist their use by others. Sensors are modelled to respond at lower-buffered concentrations of their cognate metal, compared to sensors for other effectors, explaining and correctly predicting metal specificity. However, sensors for other effectors are modelled to be only marginally less sensitive to the non-cognate metal, and indeed metal shock triggers predictable responses to non-cognate metals. Thus, we discover that tight regulation of buffered intracellular metal concentrations is a prerequisite for perfect metal specificity, rendering sensors vulnerable to dysregulation, with implications for the microbicidal action of metal fluxes.

**Results**

Sensors are selective at non-inhibitory concentrations. To compare the response of Zur, ZntR, RcnR, FrmR and FrmRE64H to Zn(II) and cobalt, *Salmonella* cells were cultured in minimal media (~4–5 h) supplemented with MNICs of each metal (giving ≤10% inhibition of growth), ensuring multiple cell division cycles in the presence of metal (Supplementary Fig. 2). Transcript abundance was visualised by end point reverse-transcriptase PCR and enumerated by quantitative PCR (qPCR; Fig. 2a–e). Expression of *znuA* was repressed upon Zn(II) supplementation, but not upon cobalt supplementation, reflecting a selective response of Zur to Zn(II) (Fig. 2a). Under the same conditions, *zntA* transcripts accumulated in response to Zn(II) but not cobalt, confirming that ZntR-mediated activation of *zntA* expression was also specific for Zn(II) (Fig. 2b). Conversely, *rcnA* transcripts accumulated in response to cobalt but not Zn(II), confirming that repression of *rcnA* by RcnR was alleviated by cobalt, but not Zn(II) (Fig. 2c). The abundance of *frm* transcripts was monitored in *Salmonella* strains containing either FrmR or variant FrmRE64H. As expected, *frm* transcripts did not accumulate in response to cobalt or Zn(II) when regulated by FrmR, but surprisingly they also failed to respond when regulated by FrmRE64H (Fig. 2d, e), despite previous observations that this variant conferred cobalt and Zn(II)-dependent β-galactosidase activity. Repression by both FrmR and FrmRE64H was exclusively alleviated by formaldehyde (Fig. 2f). Under these conditions, Zur, ZntR, RcnR and FrmR were selective for their cognate effector.

Multiple sensors respond to cobalt shock. Gene expression under the control of FrmRE64H was previously investigated via assays of β-galactosidase activity after short-term exposure to elevated cobalt: a cobalt shock. In an attempt to reconcile apparent differences between these past data and Fig. 2c, a logarithmically growing culture was exposed to cobalt for 10 min and transcript abundance visualised by end point PCR and enumerated by quantitative PCR (Supplementary Figs. 6 and 7 and Fig. 3). A twofold change in transcript abundance was used as a threshold for sensor responsiveness as indicated by arrows on quantitative PCR graphs throughout. Higher cobalt concentrations could be used during such short term, compared to prolonged, metal exposures with only a modest effect on cell viability observed (~15% reduction at 5 μM CoCl2, Supplementary Fig. 11a), while these higher cobalt concentrations are inhibitory during prolonged exposure (~30% growth reduction at 5 μM CoCl2, Supplementary Fig. 11b). Under these conditions, expression regulated by either RcnR or FrmRE64H was de-repressed by cobalt, while repression mediated by FrmR was unaffected, consistent with FrmRE64H being a cobalt-sensing variant of FrmR. However, during this transient cobalt exposure, *znuA* expression was also partially repressed relative to the control, and *zntA* expression activated (Fig. 3 and Supplementary Fig. 6), identifying both Zur and ZntR as targets of cobalt mis-metalation. The affinity of FrmRE64H for Co(II) is 500-fold weaker than that of RcnR and for this reason its response to
Cobalt shock triggers other sensors. Arrows identify the lowest exogenous [cobalt] at which each sensor appears to respond. Data for control genes are presented in Supplementary Fig. 11. Transcript abundance for the samples shown in a measured by qPCR (error bars are s.d.). Arrows represent a ≥ twofold change in transcript abundance. Heat maps of qPCR data from three biological replicates are presented in Supplementary Fig. 10.

**Fig. 3** Cobalt shock triggers other sensors. a Representative (n = 3) transcript abundance following 10 min exposure of *Salmonella* to increasing [cobalt] assayed by end point PCR. Arrows identify the lowest exogenous [cobalt] at which each sensor appears to respond. Data for control genes are presented in Supplementary Fig. 8 and full gel images in Supplementary Fig. 9. b Transcript abundance for the samples shown in a measured by qPCR (error bars are s.d.). Arrows represent a ≥ twofold change in transcript abundance. Heat maps of qPCR data from three biological replicates are presented in Supplementary Fig. 10.

### Fig. 4 Co(II) affinities of ZntR and Zur

**a** Representative (n = 3) fura-2 fluorescence emission (λex = 360 nm) upon titration of fura-2 (15.4 μM) and ZntR (9.8 μM) with Co(II). Solid line is a fit to a model describing competition from ZntR for one molar equivalent of Co(II). Dashed lines are simulated curves with K_Co(II) = 10^(-3) μM and K_Co(II) = 10^(-5) μM with Co(II).

**b** As in a but with fura-2 (14.6 μM) and Zur (9.8 μM) (n = 5).

**c** Representative (n = 3) Zur absorbance spectra upon titration of Zur (52 μM) and EGTA (50 μM) with Co(II). Dashed line is a fit to a model describing competition from Zur for two molar equivalents of Co(II). Dashed lines are simulated curves with K_Co(II) = 10^(-2) μM and K_Co(II) = 10^(-4) μM with Co(II).

Co(II) was previously considered enigmatic. However, whereas RcnR is tuned to a buffered concentration of Co(II) in cells grown in non-inhibitory cobalt concentrations, it is now evident that FrmR_E64H only responded during cobalt shock. Stepwise regulation of *Salmonella* sensors in response to increasing concentrations of cobalt shock occurred in the order RcnR, Zur, FrmR_E64H, ZntR, followed by FrmR, which did not respond (Fig. 3). It is proposed that non-cobalt sensors responded under cobalt shock because the cytosolic buffer became transiently saturated and higher intracellular concentrations occurred. This is consistent with growth inhibition during prolonged exposure to these higher metal concentrations (Supplementary Fig. 11b).

ZntR and Zur bind Co(II) with sub-micromolar affinities. We have previously demonstrated that in vitro both ZntR and Zur bind Co(II) in sites that can be displaced by Zn(II). To determine their Co(II) affinities (hereafter affinity refers to a dissociation constant), both proteins were purified following overexpression in *E. coli* and confirmed to be ≥ 95% pure (Supplementary Fig. 12) and ≥ 90% reduced. ZntR was ≥ 95% metal free, and Zur contained ~1 molar eq. Zn(II) consistent with filling of a structural Zn(II) site identified in Zur and other Fur-family members. Both proteins were competed against the fluorophore fura-2, which exhibits a decrease in fluorescence emission upon Co(II) binding and has been used to determine Co(II) affinities of metal sensor proteins including RcnR and FrmR_E64H (Fig. 4a, b). ZntR binds two Co(II) ions per dimer, and both sites were observed during competition with fura-2, with a combined affinity of 9.5 (± 1.0) x 10^-8 M (Fig. 4a and Table 1). Zur binds up to four Co(II) or Zn(II) ions per dimer in addition to the structural
Zn(II) sites (a total of 6:1 Me(II):Zur dimer)\(^{29}\). Competition with fura-2 did not distinguish between four Co(II)-binding events (Fig. 4b). In contrast, Zn(II) binding by Zur occurs with strong negative cooperativity: Sites 1–2 are tighter than site 3 by 100-fold and site 3 is tighter than site 4 by 6000-fold\(^{29}\). The intense UV-visible absorption spectra of Co(II) binding to Zur (replicated here in Supplementary Fig. 13a)\(^{29}\), allowed observable competition with the spectrally silent chelator ethylene glycol tetraacetic acid (EGTA) (Fig. 4c and Supplementary Fig. 13b), and confirmed the lack of detectable cooperativity of Co(II) binding to Zur. Data were fit to a model describing four combined Co(II)-binding events per Zur dimer (Fig. 4d). The affinity of Zur for Co(II) was determined to be 1.5 (±0.6) × 10\(^{-5}\) M averaged from both fura-2 and EGTA competition experiments (Table 1). The affinity of Zn(II) for Co(II) was comparable to that of FrmR\(^{64}\), while the affinity of Zur was approximately one order of magnitude tighter than either Zn(II) or FrmR\(^{64}\).\(^{29}\)

Co(II) affects DNA binding by Zur, FrmR\(^{64}\) and ZntR. Since Zur, FrmR\(^{64}\) and ZntR responded to cobalt shock in vivo (Fig. 3 and Supplementary Figs. 6 and 10), it seemed probable that Co(II) triggers allosteric responses which promote association of Zur with the znuA operator–promoter, dissociation of FrmR\(^{64}\) from the frmRA operator–promoter, and activation by ZntR of the zntA operator–promoter. The degree to which metal binding is coupled to DNA binding can be described as the allosteric coupling-free energy (\(\Delta G\)\(^{\text{C}}\)), which, in combination with metal affinity, contributes to metal selectivity\(^{1,7,42}\). Fluorescence anisotropy, using a fluorescently labelled dsDNA fragment containing the identified Zur-binding site upstream of znuA (Fig. 1a), was used to examine the effect of Co(II) on allostery. Initially, the stoichiometry of Zur binding to DNA was determined with saturating concentrations of Zn(II) (ensuring filling of exchangeable sites 1–4) and demonstrating that two Zn(II)-Zur dimers bind to the znuA operator–promoter sequence (Fig. 5a). E. coli Zur (93% identity to Salmonella Zur) binds to a similar target DNA sequence as two adjacent dimers with positive cooperativity\(^2\). The data for Salmonella Zur were fit to a model describing sequential binding of two Zur dimers to the znuA operator–promoter. The DNA affinity of both Zn(II)-Zur dimers was determined to be 5.4 (±1.8) × 10\(^{-8}\) M (Fig. 5b and Table 1).

| Table 1 Metal affinities, DNA affinities, allosteric coupling-free energies and abundance of Salmonella sensors |
|---|---|---|---|---|---|
| Sensor | Metal | Metal affinity (M) | DNA affinity (M) | \(\Delta G\)\(^{\text{C}}\) (kcal mol\(^{-1}\)) | Abundance (assemblies cell\(^{-1}\))\(^{3}\) |
| Zur | — | n.a. | ≥2.7 (±0.4) × 10\(^{-5}\) | n.a. | 21 (±7) |
| Co(II) | 1.5 (±0.6) × 10\(^{-8}\) | 3.1 (±0.3) × 10\(^{-8}\) | ≤−4.0 (±0.1) |
| Zn(II)
| n.a. | 5.4 (±1.8) × 10\(^{-8}\) | ≤−3.7 (±0.2) |
| Zn(II)\(^{2}\) | 6.4 (±0.4) × 10\(^{-13}\) | 4.1 (±1.0) × 10\(^{-8}\) | ≤−3.9 (±0.2) |
| ZntR | — | n.a. | 1.1 (±0.3) × 10\(^{-6}\) | n.a. | 34 (±15) |
| Co(II) | 9.5 (±1.0) × 10\(^{-8}\) | 3.4 (±1.0) × 10\(^{-7}\) | −0.7 (±0.2) |
| Zn(II) | 3.2 (±0.7) × 10\(^{-12}\) | 6.5 (±3.3) × 10\(^{-7}\) | −0.3 (±0.2) |
| RcnR | — | n.a. | 1.5 (±0.8) × 10\(^{-7}\) | n.a. | 22 (±2) |
| Co(II) | 5.1 (±0.9) × 10\(^{-10}\) | ≥1.5 (±0.2) × 10\(^{-5}\) | ≥+2.7 (±0.2) |
| Zn(II) | 9.4 (±1.0) × 10\(^{-12}\) | ≥1.3 (±0.2) × 10\(^{-5}\) | ≥+2.6 (±0.1) |
| FrmR\(^{64}\) | — | n.a. | 4.3 (±0.4) × 10\(^{-7}\) | n.a. | 149 (±4)\(^{b}\) |
| Co(II) | 2.6 (±0.4) × 10\(^{-7}\) | 2.3 (±0.3) × 10\(^{-6}\) | +1.0 (±0.1) |
| Zn(II) | 2.3 (±0.3) × 10\(^{-11}\) | 3.5 (±0.7) × 10\(^{-6}\) | +1.2 (±0.2)\(^{b}\) |
| FrmR | — | n.a. | 9.9 (±0.3) × 10\(^{-8}\) | n.a. | 135 (±17)\(^{b}\) |
| Co(II) | 7.6 (±0.4) × 10\(^{-6}\) | n.d.\(^{d}\) | n.d. | |
| Zn(II) | 1.7 (±0.7) × 10\(^{-10}\) | 3.1 (±0.4) × 10\(^{-6}\) | +2.0 (±0.1)\(^{b}\) |

All constants and abundances are means of at least triplicate determinations (n’ specified in figure legends) with ± s.d.

1 n.a. not applicable, n.d. not determined
2 This value was determined previously\(^{29}\)
3 These values were determined previously\(^{40}\)
4 A value of 2.0 × 10\(^{-5}\) M was used for mathematical modelling, estimated as described in the text

In contrast, apo-Zur (with only the structural Zn(II) sites filled) bound to the znuA operator–promoter with an affinity weaker than Zn(II)-Zur by ~500-fold (≥2.7 (±0.4) × 10\(^{-5}\) M) (Fig. 5b and Table 1). The free energy coupling metal binding to DNA binding, \(\Delta G\)\(^{\text{C}}\), for Zn(II)-Zur was calculated to be ≤−3.7 (±0.2) kcal mol\(^{-1}\). Importantly, Co(II) also promoted DNA binding by Zur with DNA affinity and \(\Delta G\)\(^{\text{C}}\) values of Co(II)-Zur determined to be 3.1 (±0.3) × 10\(^{-8}\) M and ≤−4.0 (±0.1) kcal mol\(^{-1}\), respectively (Fig. 5c and Table 1). Thus, Co(II) was as effective as Zn(II) in activating the allosteric mechanism of Zur.

FrmR\(^{64}\) was purified and biochemically characterised as described for ZntR and Zur (Supplementary Fig. 12). Fluorescence anisotropy using a fluorescently labelled dsDNA fragment containing the frmRA operator–promoter (Fig. 1d), confirmed that Co(II) triggered an allosteric response by FrmR\(^{64}\) (Fig. 5d and Table 1), such that the DNA affinity of Co(II)-FrmR\(^{64}\) was ~fivefold weaker than apo-FrmR\(^{64}\) with \(\Delta G\)\(^{\text{C}}\) + 1.0 (±0.1) kcal mol\(^{-1}\) (Table 1). Apo-ZntR bound a fluorescently labelled dsDNA fragment containing the zntA operator–promoter (Figs. 1b and 5e). At least two DNA-binding events were observed and the anisotropy change associated with the first (tightly) binding event was determined (Fig. 5e). A similar change in anisotropy (\(\Delta G\)\(^{\text{obs}}\) ≤0.025) was independently modelled for binding of an apo-ZntR dimer to the zntA operator–promoter sequence, and a DNA affinity of 1.1 (±0.3) × 10\(^{-8}\) M was determined (Fig. 5f and Table 1). An equivalent complex of Zn(II)-ZntR bound the target DNA with an affinity of 6.5 (±3.3) × 10\(^{-7}\) M (Fig. 5g and Table 1), revealing a free energy coupling metal binding to DNA binding, \(\Delta G\)\(^{\text{C}}\), for Zn(II)-ZntR of −0.3 (±0.2) kcal mol\(^{-1}\). In E. coli, ternary DNA complexes containing two ZntR dimers occur at high ZntR concentrations\(^{15}\), and additional increases in \(\Delta G\)\(^{\text{obs}}\) at elevated ZntR concentrations suggest that Salmonella ZntR forms similar ternary complexes (Fig. 5e, g). Co(II) promoted similar changes in DNA binding with affinity and \(\Delta G\)\(^{\text{C}}\) values determined to be 3.4 (±1.0) × 10\(^{-7}\) M and −0.7 (±0.2) kcal mol\(^{-1}\), respectively (Fig. 5h and Table 1). Both Co(II) and Zn(II) encouraged formation of ternary complexes, at least on this (34 bp) DNA fragment (Fig. 5g, h).

Similar numbers of ZntR, RcnR and Zur promoters per cell. The number of copies of FrmR and FrmR\(^{64}\) per cell was...
were shown. For a Co(II)-FrmRE64H data were the mean DNA affinity thermodynamically coupled such that DNA occupancy is not sensitive. However, metal binding and DNA binding are sensor, DNA affinity and tenfold lower than [B]total to ensure a surplus of available metal (buffered) concentration. 1/Kₜ was altered, iteratively, to achieve a [buffered metal] range from 10⁻³ to 10⁻¹⁶ M (Supplementary Data 1, Supplementary Software and Methods section).

RcnR was thus modelled to respond at the lowest cobalt concentration explaining why this is the bona fide sensor of Co (II) (Fig. 7). Because the weak Co(II) affinity of FrmR precluded determination of the DNA affinity for Co(II)-FrmR, this was estimated from 1/K₄ for Zn(II)-FrmR and the fold-difference between 1/K₄ for the Co(II)-FrmR₆₄₄ and Zn(II)-FrmR₆₄₄ variant (Table 1). Fractional DNA occupancy by FrmR₆₄₄ did not reach that of FrmR, due to the weaker DNA affinity of apo-FrmR₆₄₄ relative to apo-FrmR (Fig. 7). The metalated form of RcnR, as well as ZntR, activate expression by distorting their target promoter by therefore, zntAPro bound by Co(II)-ZntR [(P×M×D) was used to represent the active species. This implied a dynamic range close to that of FrmR₆₄₄ (Fig. 7). All of the other sensors were shown to be tuned above the cobalt sensitivity of RcnR, which would avoid mal-responses to Co(II). However, the margin for specificity was narrow such that ZnR would also respond to Co(II) if the concentration became an order of magnitude greater than the set point for RcnR. To create the perfect metal selectivity observed in Fig. 2, there must be the imperfect control of the intracellular cobalt concentration: Under cobalt shock, such control became imperfect (Fig. 3). Moreover, the observed sequence of activation of each sensor in response to increasing cobalt shock agreed with the order predicted by the thermodynamic models (Figs. 3 and 7): Noting that the modelled responses of FrmR₆₄₄ and ZntR overlapped (Fig. 7), although expression data indicated that the former was more sensitive to cobalt than the latter, perhaps because the effects of Co(II) on DNA binding do not fully reflect...
Zn(II) affinity of promoters decrease, co-repression by Zur occurs as occupancy of its promoter increases, and activation by ZntR occurs as the fractional occupancy of its promoter with metalated ZntR increases. Numbering reflects the order of response observed for each sensor in Fig. 3.

Thermodynamic data predict the responses to Zn(II).

FrmRE64H shares at least four metal-binding ligands with RcnR29 (Fig. 1c, d); therefore, we hypothesised that the Zn(II) affinity of RcnR may be comparable to FrmRE64H. RcnR was purified (Supplementary Fig. 12), and found to withhold one molar equivalent of Zn(II) from the spectral Zn(II)-probe magfura-2 consistent with four Zn(II)-binding sites per RcnR tetramer and an affinity at least tenfold tighter than that of magfura-2 (magfura-2 $K_{znuA} = 2 \times 10^{-8}$ M) (Fig. 8a). Consequently, to accurately determine the RcnR Zn(II) affinity, a Zn(II) probe with a tighter Zn(II) affinity of $Zn(II)-2-Zur$, was 4.1 ($\pm 0.4$) $\times 10^{-13}$ M, (Table 1), was sufficient to induce an allosteric change that enabled Zur to bind to the znuA operator–promoter. The DNA affinity of $Zn(II)-2-Zur$ was 4.1 ($\pm 1.0$) $\times 10^{-8}$ M (Fig. 8d, and Table 1). Using the Zn(II) affinities, DNA affinities and abundance of Zur, ZntR, RcnR, FrmRE64H and FrmR (Table 1), the fractional occupancy of their respective operator–promoters (with total protein or with Zn(II)-ZntR) were modelled as a function of buffered Zn(II) concentration using the same procedures as described for Co(II) (Fig. 9a). Analogous to the models for Co(II) (Fig. 7), the cognate sensors for Zn(II) were calculated to respond at the lowest buffered Zn(II) concentrations, once again explaining specificity, in this case for Zn(II) (Fig. 9a). Sensors for other effectors were tuned above this concentration, however the margin for specificity was again narrow such that RcnR would also respond to Zn(II) if the concentration became an order of magnitude greater than the set point for ZntR. To create the perfect metal selectivity shown in Fig. 2, as with Co(II), there must also be prolonged exposure (Supplementary Fig. 11d). Under these conditions, the stepwise pattern of Zn(II)-responsive gene expression, either monitored by end point PCR or qPCR, again aligned with the thermodynamic models, such that Zur responded at the lowest Zn(II) concentrations, followed by ZntR, RcnR, FrmRE64H and FrmR (Table 1), and the fractional occupancy of their respective operator–promoters (with total protein or with Zn(II)-ZntR) were modelled as a function of buffered Zn(II) concentration using the same procedures as described for Co(II) (Fig. 9a). Analogous to the models for Co(II) (Fig. 7), the cognate sensors for Zn(II) were calculated to respond at the lowest buffered Zn(II) concentrations, once again explaining specificity, in this case for Zn(II) (Fig. 9a). Sensors for other effectors were tuned above this concentration, however the margin for specificity was again narrow such that RcnR would also respond to Zn(II) if the concentration became an order of magnitude greater than the set point for ZntR. To create the perfect metal selectivity shown in Fig. 2, as with Co(II), there must also be fine control of intracellular Zn(II) concentrations.

To investigate the response of each sensor to Zn(II) shock, Salmonella cells were exposed to increasing Zn(II) concentrations for 10 min (Fig. 9b, c and Supplementary Fig. 11c). The highest Zn(II) concentrations (80 and 100 μM) were inhibitory during prolonged exposure (Supplementary Fig. 11d). Under these conditions, the stepwise pattern of Zn(II)-responsive gene expression, either monitored by end point PCR or qPCR, again aligned with the thermodynamic models, such that Zur responded at the lowest Zn(II) concentrations, followed by ZntR, RcnR, FrmRE64H and FrmR (Table 1), and the fractional occupancy of their respective operator–promoters (with total protein or with Zn(II)-ZntR) were modelled as a function of buffered Zn(II) concentration using the same procedures as described for Co(II) (Fig. 9a). Analogous to the models for Co(II) (Fig. 7), the cognate sensors for Zn(II) were calculated to respond at the lowest buffered Zn(II) concentrations, once again explaining specificity, in this case for Zn(II) (Fig. 9a). Sensors for other effectors were tuned above this concentration, however the margin for specificity was again narrow such that RcnR would also respond to Zn(II) if the concentration became an order of magnitude greater than the set point for ZntR. To create the perfect metal selectivity shown in Fig. 2, as with Co(II), there must also be fine control of intracellular Zn(II) concentrations.

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cells exert fine control over intracellular metal concentrations and after Zn(II) shock, such control becomes imperfect. The modest differential between the fractional occupancy curves for the different sensors reveals that cells are on the cusp of mis-sensing Zn(II), as well as Co(II), when subjected to metal shocks (Figs. 7 and 9a).

Discussion

Our calculations of gene regulation at different intracellular Co(II) and Zn(II) concentrations explain metal selectivity in metal sensing in terms of equilibrium thermodynamics by using determined metal affinities, DNA affinities, coupling free energies and the number of sensor molecules per cell (Figs. 7 and 9a). At first inspection, these models seem incorrect by revealing that RcnR is inherently more sensitive to Zn(II) than to Co(II) by one to two orders of magnitude (Supplementary Fig. 18), yet RcnR showed the opposite selectivity in cells during prolonged growth in elevated metal and is known to be a Co(II) sensor (Figs. 1 and 2). The explanation is that metal sensors are tuned to the buffered concentrations of their cognate metal31, and the buffered concentration of Zn(II), but not cobalt, is maintained below the set point for RcnR. The set points for the Zn(II) sensors ZntR and Zur reveal this lower buffered concentration for Zn(II) (Fig. 9a). In this context, metal specificity now becomes readily understandable by comparing the sensitivities for Zn(II) (Fig. 9a), and for Co(II) (Fig. 7), of the five sensors to reveal that the bona fide sensors are the most sensitive in the set. During prolonged growth in elevated Zn(II), the intracellular Zn(II) concentration must have been finely controlled to within about one order of magnitude in order to trigger Zur and ZntR, but not RcnR or FrmRE4H1 (Figs. 2 and 9a). This must be a buffered Zn(II) concentration, with associative metal transfer, since one hydrated ion per cell volume equates to \(-10^{-19}\) M, which would be sufficient to trigger RcnR and FrmRE4H1 (Fig. 9a). Similarly, during prolonged growth in elevated Co(II), the intracellular buffered Co(II) concentration must also have been finely controlled to within about one order of magnitude in order to trigger RcnR but not Zur (Figs. 2 and 7). Thus, these metal sensors are adapted to discriminate perfectly between these inorganic elements only when metals are buffered, with associative metal transfer, and when metal concentrations are finely controlled.

The models predict that if the buffer becomes saturated then the Zn(II) sensors will respond to Co(II) and vice versa the Co(II) sensor will respond to Zn(II) (Figs. 7 and 9a). During Zn(II) shock, FrmRE4H1 and RcnR did respond, consistent with the Zn(II) concentration having transiently increased above the buffered concentration (Fig. 9b, c). Similarly during Co(II) shock Zur, FrmRE4H1 and ZntR responded (Fig. 3). For both metals, the order of the response to increasing metal shock (Fig. 3 and 9b, c), correlated with the order predicted from the thermodynamic properties of the sensors (Figs. 7 and 9a), further validating the models and suggesting that even the shock responses are not solely determined by on-rates (kinetics). This also reveals that when assigning metal specificity to metal sensors by monitoring gene expression in cells exposed to exogenous metals, care should be taken to optimise the growth conditions and avoid saturation of the intracellular buffer. For sensors with more than one DNA target, multiple set points may exist to allow a graded response to changing metal demands as the cytosolic metal buffer becomes increasingly full. Intriguingly, Bacillus subtilis Zur has at least three set-points reflecting filling of its multiple Zn(II) sites and its varying DNA affinities on different operator–promoters, giving rise to three waves of Zn(II)-dependent gene expression31. These three waves could reflect different levels of saturation of the cytosolic buffer. Alternatively, evidence of mal-responses of sensors for other metals might indicate if one of the waves occurs when the buffer becomes fully Zn(II) saturated.

The crowded cytosol contains a multitude of sulphur, nitrogen and oxygen ligands associated with an array of metabolites and macromolecules, many of which can be readily organised into different metal-binding combinations and geometries. Such a polydisperse mixture will inevitably bind and buffer metals in the order of the Irving Williams series41. It is anticipated that cytosol-facing metal-binding sites of metal transporters will also be tuned to these buffered metal concentrations. For some metals, and in some organisms, the cytosolic buffer may be dominated by a single molecule such as glutathione or its substitutes such as bacillithiol, or a free amino acid such as histidine41, 52–53. Macromolecules with multiple labile metal sites, e.g., metallothioneins, may also be induced to expand the depth of the buffer when metals such as Zn(II) increase in abundance56. At present, it is unclear whether or not the buffer for either Co(II) or Zn(II) in Salmonella is dominated by a single, and potentially shared, molecule. Notably, mutants deficient in the synthesis of
glutathione showed impaired detection of Co(II) and Zn(II) by FrmR,
but now we know that metal detection by this variant sensor only occurs during metal shock, presumably once components of the bona fide buffer have become saturated. In contrast, there is negligible effect of glutathione on Zn(II) sensing by Salmonella ZntR,
whereas Zn(II) sensing by S. subtilis CzrA is enhanced in the absence of bacillithiol. It is noteworthy that Salmonella is at least an order of magnitude more sensitive to exogenous cobalt than Zn(II) (Supplementary Fig. 1). Unlike E. coli, Salmonella requires cobalt to synthesise cobalamin, vitamin B12, but this is only made under anaerobic conditions which perhaps renders Salmonella proteins susceptible to mismetabolization by unwanted, un-sequestered, cobalt during aerobic growth. In eukaryotes, there is scope for diversity in buffered metal concentrations within different intracellular compartments (nucleus, organelles, vesicles, trans-Golgi network, endoplasmic reticulum, for example) and spectral probes have been developed to interrogate such concentrations. Since metal-sensing transcriptional regulators can also report upon metal occupancy in vivo and are tuned to metal concentrations for example in the bacterial cytosol, then their metal sensitivities provide an alternative approach to interrogate the vital buffered metal concentrations: In short, the K₅ values at which each sensor responds using the calculations described here (Fig. 6, Supplementary Data 1, Supplementary Software and Methods section). In the same way that metal selectivity of metal sensors becomes comprehensible in the context of these values (Figs. 7 and 9a and Supplementary Fig. 18), so metalation of other metalloproteins should become understandable once a complement of cellular K₅ values for different metals have been calculated.

In conclusion, we discovered that perfect metal specificity in metal sensing was restricted to a finely controlled range of buffered metal concentrations, which were exceeded during metal shocks (Figs. 3, 7 and 9). These data support the prediction that bacteria are susceptible to the mis-sensing of metals and hence the notion that this vulnerability is exploited by immune systems. Metals, chelants and ionophores also have a long history of use as antimicrobials in medicine, agriculture and as preservatives. However, the development of this wide spectrum of metal related treatments has largely been empirical, up to now.

Methods
Bacterial strains, DNA manipulations and growth conditions. 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), and originally from the Salmonella Genetic Stock Centre. Deletion derivative ΔfrmR (SL1344 lacking the frmR coding sequence) was generated previously. E. coli strain DH5α was used for routine cloning and strain BL21(DE3) was used for recombinant protein overexpression (both from historical lab stocks). E. coli strains BW25113ΔzntR::kan (JW3254-2) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously. E. coli strains BW25113ΔzntR::kan (JW3254-5) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously. E. coli strains BW25113ΔzntR::kan (JW3254-5) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously. E. coli strains BW25113ΔzntR::kan (JW3254-5) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously. E. coli strains BW25113ΔzntR::kan (JW3254-5) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously. E. coli strains BW25113ΔzntR::kan (JW3254-5) and BW25113ΔnikR::kan (JW3446-3) were originally from the Keio collection (and a gift from D. Weinkove, Durham University). Kanamycin resistance cassettes were removed using the helper plasmid pCP20 carrying the FLP recombinase. Promoter-lacZ fusion constructs with the frmR operator-promoter and frmR or frmRΔ coding sequence upstream of lacZ, have been described previously.

Generation of E. coli BW25113 double-deletion mutants. BW25113Δzur::cat was generated by the λ Red method, using plasmid pKD3 and primers 1 and 2 (Supplementary Table 4; hereafter all primer numbers relate to this table). Mutants
were selected on LB medium supplemented with chloramphenicol. The Δznt::cat fragment was moved into strain BW25113ΔzntR (kan cassette removed) by P1 transduction. The chloramphenicol resistance cassette was removed and genotype confirmed by PCR using primers 3–6. P1 transducing lysate from BW25113ΔzntR:: kan ( JW2902-1, a gift from P. Chivers, Durham University) was used to move the ΔzntR::kan fragment into BW25113ΔzntR kan cassette removed. The kan cassette was removed and genotype confirmed by PCR using primers 7–10.

RNA extraction and reverse-transcriptase PCR. Expression mediated by FrmR and FrmRE64H was measured in Salmonella strain SL1344ΔfrmR-containing either pPtmA::frmR or pPtmA::FrmRE64H reporter constructs (generating SL1344FrmR and SL1344FrmRE64H, respectively) cultured in supplemented M9 minimal medium following dilution of overnight cultures to an OD of 0.25 at 600 nm. To enable direct comparison of metal sensor responses with FrmR and FrmRE64H-mediated expression, expression experiments were conducted in 60 mM NaCl, 240 mM KCl, 10 mM HEPES pH 7.0 with addition of 1.2 molar equivalents of ZnCl2 (per monomer). ZnR, ZnR and FrmR and FrmRE64H were prepared in 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0, with addition of 2.2 molar equivalents ZnCl2 for ZnR (per monomer) for ZnR-Zur, which saturates the four exchangeable sites per dimer, 1 molar equivalent ZnCl2 for ZnR-Zur, which only saturates two of the sites, 2.2 molar equivalents CoCl2 for Co(II)-Zur, 1.2 molar equivalents ZnCl2 or CoCl2, for ZnR-Zur or CoR-Zur, respectively, or 100 μM CoCl2 for CoR-FrmR and FrmRE64H.

Transcript abundance by end point and quantitative PCR. Transcript abundance by end point and quantitative PCR was performed as described40, by titration of ZnR-Zur into 1 μM znuA or znuA–zntA–rcnA–dnaB DNA binding affinities determined using 10 nM dsDNA probe. DNA affinity and coupling free energies (ΔG°) were determined with Dynafit29, 40.7. 40. All oligonucleotides are listed in Supplementary Table4. Competitor DNA for in vitro RNA quantitation, soluble cell lysates were prepared in 40 mM NaCl, 160 mM KCl, 10 mM HEPES pH 7.0, 10 mM DTT, pH 7.8, with inclusion of 1.2 molar equivalents DTT (per monomer) for ΔzntA–znuA, ΔznuA, ΔrcnA, ΔzntA–znuA–rcnA, ΔzntA–znuA–rcnA–dnaB. A quadratic 1/x2 weighted regression model was used to fit data in Figure legends. Fura-2 divalent metal titrations were conducted in 20 μM Fura-2 in supplemented M9 minimal medium (0.25 μM CoCl2, 50 μM ZnSO4 described above; Supplementary Fig. 11), and 50 μM formamidine (determined under the same conditions).40. For metal shock exposures, logarithmic cells were statically cooled to 25 °C for 20 min followed by a 10 min exposure to CoCl2 or ZnSO4. An aliquot (1.2 ml) of culture was used for RNA extraction using RNAeasy Protect Bacteria Mini Kit (Qiagen) as described29. DNA was purified from RNA with ethanol precipitation and treated with DNase I (Fermentas; 1 U per 44 ng RNA), and 300 ng RNA used per reverse transcriptase reaction (Invitrogen™ Reverse Transcription System, Promega). Negative controls without reverse transcriptase were performed in parallel.

Protein overexpression and purification. E. coli BL21(DE3) containing pETzur, pETFrmR and pETRcnR was used to overexpress ZntR, Zur, FrmRE64H and RcnR, respectively26. Protein purification was conducted using a combination of Ni(II) affinity, gel filtration, heparin affinity and ion-exchange chromatography26. Experimentally determined extinction coefficients were used to quantify purifications, which were confirmed to be 95% pure as assessed by SDS-PAGE (Supplementary Fig. 12). Ananerobic protein stocks (maintained in an anaerobic chamber) were prepared as described and confirmed to be 95% metal free and 98% reduced26, with the exception of Zur which contained ~1 molar equivalent of Zn(II) (per monomer) as purified. In all in vitro experiments, protein stocks were incubated under anaerobic conditions using N2-purged buffers29.

Determination of metal affinities. All experiments were conducted in 100 mM NaCl, 400 mM KCl, 10 mM HEPES pH 7.0. For competition with fura-2, CoCl2 was titrated into a mixed solution of protein and fura-2 and fluorescence emission was recorded at equilibrium at 510 nm (λex = 360 nm; T = 20 °C) using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies)29. Fura-2 was quantified using the extinction coefficient ε380 nm = 28,000 M–1 cm–1. For competition with EGTA, CoCl2 was titrated into a mixed solution of Zur and EGTA, and absorption spectra were recorded at equilibrium using a λex UV-visible spectrophotometer (Perkin Elmer Life Sciences). Control experiments without EGTA were also performed. For competition with EGTA, CoCl2 was titrated into a mixed solution of Zur and MgCl2 or ZnCl2 and quin-2, and absorbance was recorded at equilibrium at 325 nm (magnfura-2) or 650 nm (quin-2). Magfura-2 and quin-2 were quantified using the extinction coefficients ε380 nm = 22,000 M–1 cm–1 and ε360 nm = 37,000 M–1 cm–1, respectively. Competition data were fit to a model described in figure legends and Table 1 using Dynafit29 and determined Co(II) and Zn(II) affinities26. Mean and standard deviation values were determined from at least triplicate analyses (n′ specified in figure legends). Fura-2 KD50 = 8.64 ± 10–8 M at pH 7.0. EGTA KD50 = 7.89 ± 10–8 M. At pH 7.0 determined using Schwarzenbach’s α=coefficient method26. Magfura-2 KZn(II) = 2 × 10–8 M at pH 7.0 and quin-2 KD50 = 3.7 ± 10–8 M.

Fluorescence anisotropy. Fluorescently labelled double-stranded DNA probes, znuAPro and zntAPro were generated using oligonucleotides 23 (hexachloro-fluorocin labelled) and 24 and containing the identified Zur binding site upstream of znuA, or 25 (hexachloro-fluorocin labelled) and 26 containing the identified ZnR binding-site upstream of zntA, frmRAp and rcnAPro have been described previously40. All oligonucleotides listed in Supplementary Table 1 using Dynafit and the script in Supplementary Software, using determined metal affinities (1/Kd), DNA

Quantitation of protein abundance. E. coli strains BW25113ΔzntR Δzur and BW25113ΔzntR Δzur ΔrcnR, and Salmonella strain SL1344 were cultured to logarithmic phase in supplemented M9 minimal medium. Purified stocks of Zur, ZnR and RcnR were quantified by amino acid analysis (UC Davis). For ZnR and Zur quantitation, soluble cell lysates were prepared in 40 mM NaCl, 160 mM KCl, 10 mM HEPES pH 7.0, 1 mM DTT, pH 7.8, with inclusion of 1.2 molar equivalents DTT (per monomer) for ΔznuA, ΔrcnA, ΔzntA–znuA–rcnA, ΔzntA–znuA–rcnA–dnaB. The anisotropy change associated with a dimer binding to DNA was determined to be 0.025 by using Dynafit to simultaneously fit the data from apo-Znr titrations (n = 7, Fig. 5f) and this value was then fixed to individually fit the data sets for apo-, ZnR- (II)- and Co(II)-ZntR and determine DNA affinities. Mean and standard deviation values were determined from at least triplicate analyses (* specified in figure legends).
affinities (1/K_i and 1/K_p) plus cellular abundance of each sensor (P) and DNA target (D) (Table 1)). Where the standard deviations for the DNA affinities of Co (II)– and Zn(II)–bound proteins overlapped, average values generated by combining the data for both metals were used for 1/K_i. These were: 3.6 × 10^{-8} M for Co (II)-Zur and Zn(II)-Zur, 1.4 × 10^{-7} M for Co(II)-RcnR and Zn(II)-RcnR, and 4.7 × 10^{-7} M for Co(II)-ZntR and Zn(II)-ZntR. To determine the amount of (P)(M) or (P)(Zn) for Zur, the response for PD was removed from the Dyna script. A cell volume of 1 fl was used to calculate [P] and [P]_cell from the number of protein assemblies per cell (i.e. dimers or tetramers) (Table 1) and target DNA binding sites per cell (assumed to be 1 copy per cell for RcnR and ZntR, 4 copies per cell for Zur due to additional gene targets, and 15 copies per cell for FrmR and FrmRE64H). The following are step-by-step instructions to calculate fractional DNA occupancy as a function of buffered metal concentration [(column ‘M’)] and adjust the column ‘N’ as described in step 8. (10) Plot fractional DNA occupancy (column ‘N’) occupancy by a metal sensor as a function of buffered metal concentration, with due to additional gene targets, and 15 copies per cell for FrmR and FrmRE64H. Due to the additional gene targets, and 15 copies per cell for FrmR and FrmRE64H.

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